A phytochemical and pharmacological investigation of Momordica charantia Linn. fruit with respect to its reputed anti-diabetic properties

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A PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF *MOMORDICA CHARANTIA* LINN. FRUIT WITH RESPECT TO ITS REPUTED ANTI-DIABETIC PROPERTIES

A thesis submitted by

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Abstract

Plants have been used traditionally for the treatment of diabetes mellitus all over the world. One such example is the bitter-tasting unripe fruit of *Momordica charantia* Linn. (Cucurbitaceae), commonly known as karela.

Numerous studies in humans, animals and *in vitro* models have demonstrated a potential beneficial effect of karela juice or extracts in diabetes. However, the mode of action and active components of karela have not been satisfactorily explained. The present study aimed to use a systematic, relevant approach (bioassay-guided fractionation) to isolate and identify orally active anti-diabetic phytochemicals in karela, and to elucidate its mode of action.

Our results showed that karela from different geographical origins varied in both their physical appearance and chemical constituents. Two varieties (Thai and Kenyan) of karela juice exhibited anti-hyperglycaemic (significant improvement in oral glucose tolerance) but not hypoglycaemic (no effect on basal glycaemia) activities in streptozotocin-induced non-insulin-dependent diabetic (NIDD) rats. Thai karela, being more potent, was studied further. We observed for the first time *in vivo* insulin secretagogue activity by karela juice. The water extract of karela juice significantly improved oral glucose tolerance but not intraperitoneal or intravenous glucose tolerance, implying that it inhibits glucose absorption from the gut or influences glucose sensitive gut hormones. The hexane extract improved oral and intraperitoneal but not intravenous glucose tolerance suggesting that it may possess a hepatic insulin sensitising effect, possibly in addition to an effect on the gut. This extract also inhibited *in vitro* intestinal glucose absorption in a brush border membrane vesicle model.

Bioassay-guided fractionation of the hexane extract led to the isolation and identification of 3 compounds: 5,25-stigmastadien-3-ol (approx. 0.15%w/w yield), momordicine I (0.05%w/w) and β-sitosterol. Both 5,25-stigmastadienol and β-sitosterol were shown to improve oral glucose tolerance at doses of 4.6mg/kg and 3.1mg/kg respectively in NIDD rats. Momordicine I was not tested.

We concluded that karela is most likely to contain more than a single anti-diabetic component and it exhibits its anti-hyperglycaemic activity via a combination of modes of action.

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Abbreviations and symbols

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BBMV	brush border membrane vesicles
BDA	British Diabetic Association
BNF	British National Formulary
C	Celsius
^{13}C NMR	carbon-13 nuclear magnetic resonance
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
$CDCl_3$	deuterochloroform
C_6H_{14}	hexane
$CHCl_3$	chloroform
CH_3CN	acetonitrile
Cl	chloride; chlorine
cm	centimeter
COSY	correlated spectroscopy
cpm	counts per minute
DEPT	distortionless enhancement by polarization transfer (differentiation of CH, CH_2 and CH_3)
dl	decilitre (= 100ml)
DNA	deoxyribonucleic acid
EIMS	electron impact mass spectrometry
EtOAc	ethyl acetate
FABMS	fast atom bombardment mass spectrometry
FCPD	fibrocalculous pancreatic diabetes
Fig.	figure
g	gram
ΔG	the sum of the increase in plasma glucose at all time points compared with time 0
G-6-PDH	glucose-6-phosphate dehydrogenase
GDM	gestational diabetes mellitus

Abbreviations and symbols

GLP-1	glucagon-like peptide 1
Hb	haemoglobin
h/hr	hour
^{3}H	tritium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte group A
^{1}H NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
Hz	hertz
IC_{50}	median inhibitory concentration
IDDM	insulin-dependent diabetes mellitus
IGT	impaired glucose tolerance
IPGTT	intraperitoneal glucose tolerance test
IVGTT	intravenous glucose tolerance test
J	nuclear spin-spin coupling constant (in Hz)
K^+	potassium ion
KCl	potassium chloride
kg	kilogram
L	litre
LD_{50}	median lethal dose
Lys	Lysine
M	molar
M^+	molecular ion
MCL	*Momordica charantia* lectin
MeOH	methanol
Mg	magnesium
$MgSO_4$	magnesium sulphate
mg	milligram
MHz	megahertz
min	minute

Abbreviations and symbols

ml	millilitre
mM	millimolar
mmol	millimole
M. wt	molecular weight
MRDM	malnutrition-related diabetes mellitus
MS	mass spectrometry
m/z	mass to charge ratio
Na	sodium
Na^+	sodium ion
NaCl	sodium chloride
NaSCN	sodium thiocyanate
NAD	nicotinamide adenine dinucleotide
NIDDM	non-insulin-dependent diabetes mellitus
nM	nanomolar
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
NOESY	nuclear overhauser enhancement spectroscopy
ODS	octadecyl silica
OGTT	oral glucose tolerance test
pH	hydrogen ion concentration
pI	pH of a solution containing a solute at its isoelectric point
pmoles	picomoles (10^{-12})
ppm	parts per million
Pro	Proline
psi	pounds per square inch
PTLC	preparative thin layer chromatography
rpm	revolutions per minute
R_f	numerical value (0-1) of $\frac{\text{Distance from baseline to centre of spot}}{\text{Distance from baseline to solvent front}}$
R_t	retention time
s/sec	second
SEM	standard error of mean

Abbreviations and symbols

STZ	streptozotocin
TLC	thin layer chromatography
TMS	tetramethylsilane
Tris	2-amino-(hydroxymethyl)propane-1,3-diol
U	unit
UV	ultraviolet
v/v	volume per volume
WHO	World Health Organisation
w/v	weight per volume
w/w	weight per weight
µ	micro-; micron
µg	microgram
µl	microlitre
µm	micrometer
α	alpha
β	beta
γ	gamma
λ	lambda
δ	chemical shift (in ppm)
/	per
<	less than
>	more than
≤	less than or equal to
≥	more than or equal to
±	plus or minus
%	percentage

Details of publications

Refereed paper

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(A poster presented at the 32nd annual meeting of the European Association for the Study of Diabetes held at the Austria Center Vienna, Vienna, Austria; 1-5 September 1996)

Oral communications

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by **Lau C**, Raman A, Noel M, Kergoat M, Lawrence J and Dodoo A.

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Title: A fruit juice for diabetics
by **Lau C**.

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Posters

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Title: The anti-hyperglycaemic activity of the unripe fruit of *Momordica charantia* L.
by **Lau C**, Raman A, Noel M, Kergoat M and Autier V.

A poster presented at Plants for Food & Medicine held at Imperial College London, UK. (1-5 July 1996)
Title: Variation in constituents and anti-hyperglycaemic activity of *Momordica charantia* Linn. of different origins
by **Lau C**, Raman A, Noel M and Kergoat M.

Chapter 1:

Introduction

Chapter 1: Introduction

1.1 Diabetes mellitus

According to the World Health Organisation estimates (Leslie, 1992), there are at least 120 million people world-wide suffering from diabetes mellitus. In the UK, around 1.4 million people are known to have diabetes, which accounts for 2% of its population (BDA, 1995). "Diabetes mellitus", which comes from the Greek and Latin words for syphon and sugar, describes one of the symptoms of uncontrolled diabetes - the passing of a large amount of urine containing sugar (glucose). In fact, it is a chronic metabolic disorder characterised by a high blood glucose concentration, known as hyperglycaemia (a fasting venous plasma glucose $\geq$ 7.8 mmol/L), which is due to insulin deficiency and/or insulin resistance (defective cellular insulin action). The characteristic symptoms include excessive thirst and hunger, muscular weakness, excessive urination (polyuria) and glycosuria (glucose is excreted in the urine when the renal threshold for its reabsorption is exceeded), and sometimes weight loss. The metabolic disturbance involves the metabolism of carbohydrates, fat and protein. As a consequence of the metabolic derangements in diabetes, various complications often develop which result in a considerably reduced life expectancy. These complications are mainly due to diseases of blood vessels, either macrovascular disease which leads to an increased prevalence of coronary artery disease and peripheral vascular disease, or microvascular damage which contributes to diabetic nephropathy and diabetic retinopathy. Other potential complications include foot trauma and ulceration, and increased susceptibility to infection (Gale, 1990; Nathan, 1993).

The current classification and diagnostic criteria for diabetes mellitus were introduced by the United States National Diabetes Data Group (1979) and with some modifications by the World Health Organisation (WHO) (1980, 1985 and 1994). Diabetes mellitus can be categorised into various types: insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM) which is subdivided into obese NIDDM and non-obese NIDDM, malnutrition-related diabetes mellitus (MRDM), diabetes secondary to pancreatic disease, e.g. chronic pancreatitis, haemochromatosis and pancreatectomy, diabetes secondary to endocrine disease, e.g.

Cushing's syndrome, acromegaly and phaeochromocytoma, drug-induced diabetes caused by the administration of certain drugs such as tricyclic antidepressants and thiazides, diabetes associated with genetic syndromes, e.g. Friedreich's ataxia and myotonic dystrophy, impaired glucose tolerance (IGT) and gestational diabetes mellitus (GDM). Some of these are described in more detail below.

1.1.1 Insulin-dependent diabetes mellitus (IDDM)

About one-fifth of all diabetic sufferers are insulin-dependent. IDDM or Type I diabetes is usually characterised clinically by abrupt onset of symptoms, insulinopenia and dependence on exogenous insulin therapy for survival, and proneness to ketosis and ketoacidosis. Patients with insulin dependent diabetes are virtually devoid of endogenous insulin secreting activity (with an absolute insulin deficiency), which results from the extensive destruction of beta cells from the islets of Langerhans in the pancreas. The loss of β-cells may be due to exogenous chemicals from the environment, viral infection (with Coxsackie or Echo virus), or immunological factors such as an autoimmune disorder. IDDM patients are usually young and not obese when they first develop symptoms. IDDM is also strongly associated with histocompatibility antigens HLA-DR3 (B8, B18) or HLA-DR4 (BW15) on chromosome 6 (Cudworth and Woodrow, 1976; Gale, 1990; Vadheim and Rotter, 1992).

1.1.2 Non-insulin-dependent diabetes mellitus (NIDDM)

The majority (80%) of diabetic sufferers are non-insulin-dependent. Unlike IDDM where the primary defect is lack of insulin production, in NIDDM (also referred to as Type II diabetes), there are both hepatic and peripheral insulin resistance, i.e. decreased insulin effectiveness (Turner, 1996) and impaired insulin secretion (Robertson, 1992). Insulin resistance is characterised by impaired uptake and utilisation of glucose in insulin-sensitive target organs such as adipocytes and skeletal muscle, and by impaired inhibition of hepatic glucose output. Thus normal glycaemic control is maintained only if the pancreatic β-cells can increase their insulin-secreting capacity to compensate for the extent of insulin resistance. Thus in many patients with impaired glucose tolerance, insulin resistance is also associated with

hyperinsulinaemia. Unlike IDDM, patients with NIDDM are not dependent on insulin for prevention of ketonuria and are not prone to ketosis. The onset is typically in later life (usually after age 40), and it is often associated with obesity (approximately 80% of affected individuals are obese). NIDDM also has a stronger genetic basis than in IDDM, however, characteristic aggregation of HLA types and islet cell antibodies have not been found in this type of diabetes.

1.1.3 Malnutrition-related diabetes mellitus (MRDM)

This distinct variety of diabetes is only found in tropical developing countries, thus it is also known as tropical diabetes (Gale, 1990). As the name implies, the diabetic sufferers of this group have histories of severe malnutrition. In addition, it is associated with insulin dependence, sometimes with severe but fluctuating insulin resistance. One of the two subclasses, fibrocalculous pancreatic diabetes (FCPD), is associated with exocrine pancreatic deficiency, pancreatic fibrosis and calcification, and the presence of stones in the pancreatic duct. The cause is believed to be related to the consumption of foods containing cyanogenic glycosides, such as cassava (*Manihot esculenta* Crantz, Euphorbiaceae), which may result in pancreatic damage. The other subclass, protein-deficient pancreatic diabetes, appears to be a direct consequence of early childhood malnutrition, such as kwashiorkor in which β-cell damage occurs.

1.1.4 Impaired glucose tolerance (IGT)

The diagnosis of IGT is established by glucose concentrations during an oral glucose tolerance test (OGTT). After an overnight fast, 75g of glucose is taken in 250-350ml of water. Blood samples are taken in the fasting state and 2 hours after glucose administration. IGT is defined solely by the abnormal glucose values during an OGTT that lie between the normal and diabetic ranges, i.e. the fasting venous plasma glucose concentration is below 7.8mmol/L and the 2-hour OGTT value is between 7.8 and 11.1 mmol/L (National Diabetes Data Group, 1979).

While individuals in this class are not considered to be diabetic, they are at higher risk than the general population for the development of diabetes. IGT probably represents a key stage in the natural history of NIDDM (and less frequently of IDDM), and it can

be expected that 1-5% of persons with IGT will proceed to overt clinical diabetes per year. Subjects with IGT have a heightened risk of macrovascular disease (Harris and Zimmet, 1992) and because of this and the association with other cardiovascular disease risk factors including hypertension, dyslipidaemia and central obesity, the diagnosis of IGT particularly in otherwise healthy individuals, may have important prognostic implications. IGT is widely accepted as a precursor of NIDDM (Edelman, 1995).

1.1.5 Gestational diabetes mellitus (GDM)

The term gestational diabetes mellitus is restricted to pregnant women in whom the onset or first recognition of glucose intolerance occurs during pregnancy and the life-time risk for IGT and NIDDM is substantially increased (WHO, 1994). Thus, diabetics who become pregnant are not included in this class. GDM is associated with increased perinatal complications and with increased risk for progression to diabetes within 5-10 years after parturition (Harris and Zimmet, 1992).

Many women with GDM may be treated with diet alone or, if necessary, also with insulin. Oral hypoglycaemic agents are avoided due to the potential risk to the foetus.

1.1.6 Management of diabetes mellitus

The therapeutic goal for diabetes is initially to relieve the immediate symptoms of the disorder (thirst, polyuria, glycosuria and weight loss) without causing hypoglycaemia, and in the long term, to prevent diabetic complications and reduce the mortality of diabetes.

Monitoring of therapy is the key to management of diabetes. The monitoring of blood glucose has generally superseded the detection of glycosuria, though urine testing for glucose is useful in patients who have difficulty in blood glucose monitoring. For adequate glycaemic control, the aim is to reduce fasting blood glucose concentrations to within the range of 3.3 to 5.6 mmol/L venous whole blood, while postprandial blood glucose concentrations should be below 10 mmol/L. Blood glucose concentration evaluation can be carried out using blood glucose reagent test strips which can be read by visual comparison with a colour chart, or automatically by means of a meter (BNF, 1998). In addition, detection of urinary ketones is useful in

diabetic patients prone to ketosis; and the degree of haemoglobin glycosylation (HbA_1 or) HbA_{1c}) is used as an indicator of mean glycaemic control over the preceding two to three months.

All diabetic patients require careful consideration of the diet. Each patient requires an individually tailored diet plan according to his or her needs, taking account of the patient's usual eating habits, lifestyle, work patterns and culture. The typical dietary advice includes: not restricting carbohydrate intake, but meals should consist of unrefined carbohydrates (starch, soluble fibre) instead of simple sugars such as sucrose, increasing dietary fibre, and reducing saturated fat intake (Lean *et al.*, 1992; BDA, 1996). Correction of obesity is desirable in NIDDM patients, since it will remove one of the factors associated with insulin resistance. Moreover, all diabetic patients are encouraged to exercise. Exercise improves metabolism and enhances the action of insulin on tissues (Devlin and Horton, 1987).

1.1.7 Insulin and its use in diabetes mellitus

Insulin is the main form of treatment for patients with IDDM, who have little or no endogenous insulin secretory capacity. Since its discovery in 1922 (Banting *et al.*, 1922), insulin has provided life-saving treatment for millions of diabetic patients around the world. Insulin (Rang *et al.*, 1995; Laurence *et al.*, 1997) is a peptide macromolecule (composed of 51 amino acid residues with approximate molecular weight of 6000 daltons) consisting of two peptide chains (A chain, 21 and B chain, 30 amino acids) connected by two disulphide bridges. It is derived by proteolytic cleavage from a larger single-chain protein precursor known as proinsulin of approximate 9000 daltons molecular weight (Fig. 1.1). Insulin is synthesised in the β-cells of the pancreatic islets in the form of proinsulin, which is stored in secretory granules. A peptide fragment known as connecting C-peptide breaks off from proinsulin during the secretory process (by exocytosis), so that equimolar quantities of insulin and C-peptide are released into the circulation. Insulin enters the portal circulation and eventually reaches the liver which is its prime target organ. About 50% of secreted insulin is extracted and degraded in the liver; the remaining is broken down by the kidney.

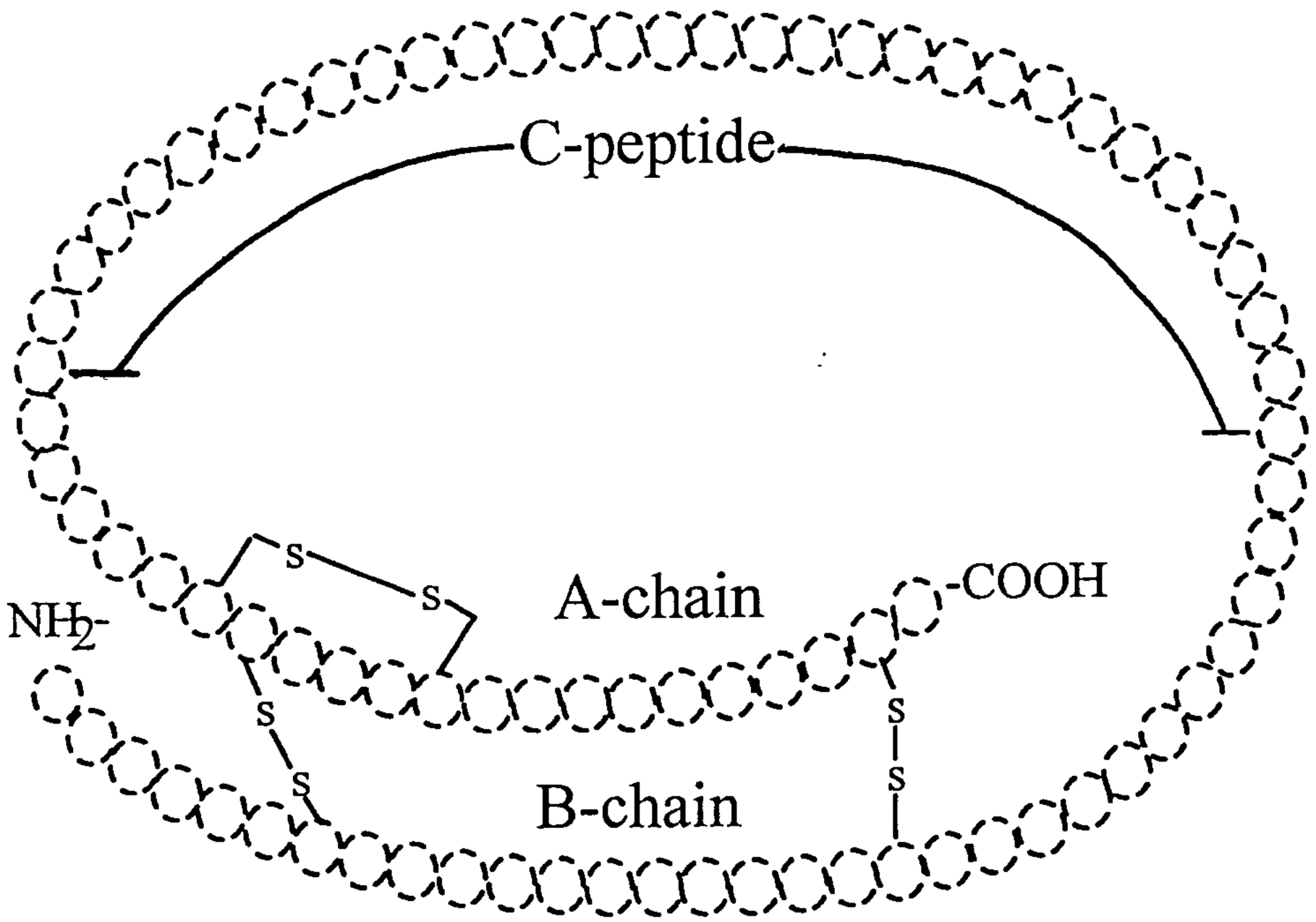


Figure 1.1: The proinsulin molecule

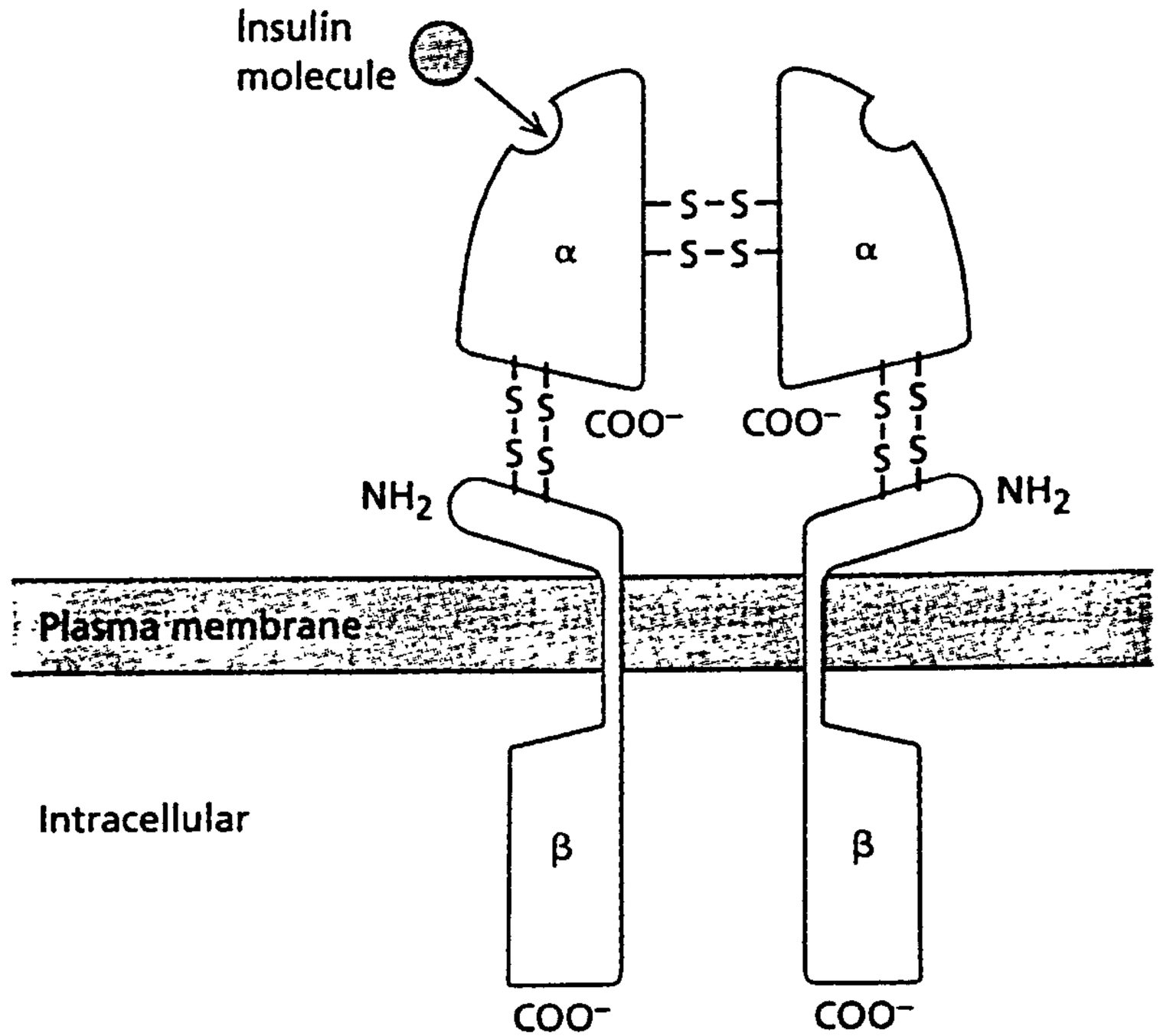


Figure 1.2: The insulin receptor consists of α and β subunits linked by disulphide bridges.
(Reproduced from "Clinical Medicine", Kumar PJ and Clark ML; Bailliere Tindall, UK, 1990)

The insulin receptor (Fig. 1.2), a glycoprotein of approximate 350000 daltons molecular weight, is present on many target cells (Gale, 1990). The receptor straddles the cell membrane and consists of a dimer with two α subunits, which include the binding sites for insulin, and two β subunits, which transverse the cell membrane and initiate the intracellular actions of insulin. Insulin molecules bind to the receptors, forming a complex that promotes glucose uptake.

About 25% of total pancreatic insulin content is secreted daily. The main parameter controlling the synthesis and secretion of insulin is the blood glucose concentration. In normal subjects, there is a steady basal insulin release and also a response to a rise in blood glucose. It is a two-phase response: an initial rapid phase (first phase) reflecting release of stored insulin, and a slower, delayed phase (second phase) reflecting the release of both stored insulin and new synthesis (Pfeifer *et al.*, 1981; Rang *et al.*, 1995). However, this response is abnormal in diabetic patients; the first phase is missing in NIDDM and both phases are missing in IDDM (Fig. 1.3). Apart from glucose, the first phase insulin release can also be stimulated by glucagon, amino acids (particularly arginine and leucine), fatty acids, various gastrointestinal tract hormones (gastrin, secretin, cholecystokinin, gastric inhibitory peptide and enteroglucagon) and sulphonylureas (Section 1.1.9(a)). On the other hand, insulin release is inhibited by somatostatin and amylin.

Insulin is the major regulator of intermediary metabolism (Rang *et al.*, 1995). It exerts its effects mainly on the carbohydrate, fat and protein metabolism in liver, muscle and adipose tissues (Table 1.1). Its most obvious effect is to reduce blood glucose by decreasing glycogenolysis (glycogen breakdown) and gluconeogenesis (synthesis of glucose from non-carbohydrate source) and increasing glycogen synthesis. It also increases glucose utilisation (glycolysis), fatty acid and amino acid synthesis, and decreases lipolysis.

The aim of insulin therapy is to achieve the best possible control of blood glucose concentrations without the risk of hypoglycaemia. Insulin may be of bovine or porcine origins, or it can be human insulin produced by either enzymatic modification and suitable purification of porcine insulin or by recombinant DNA technology using

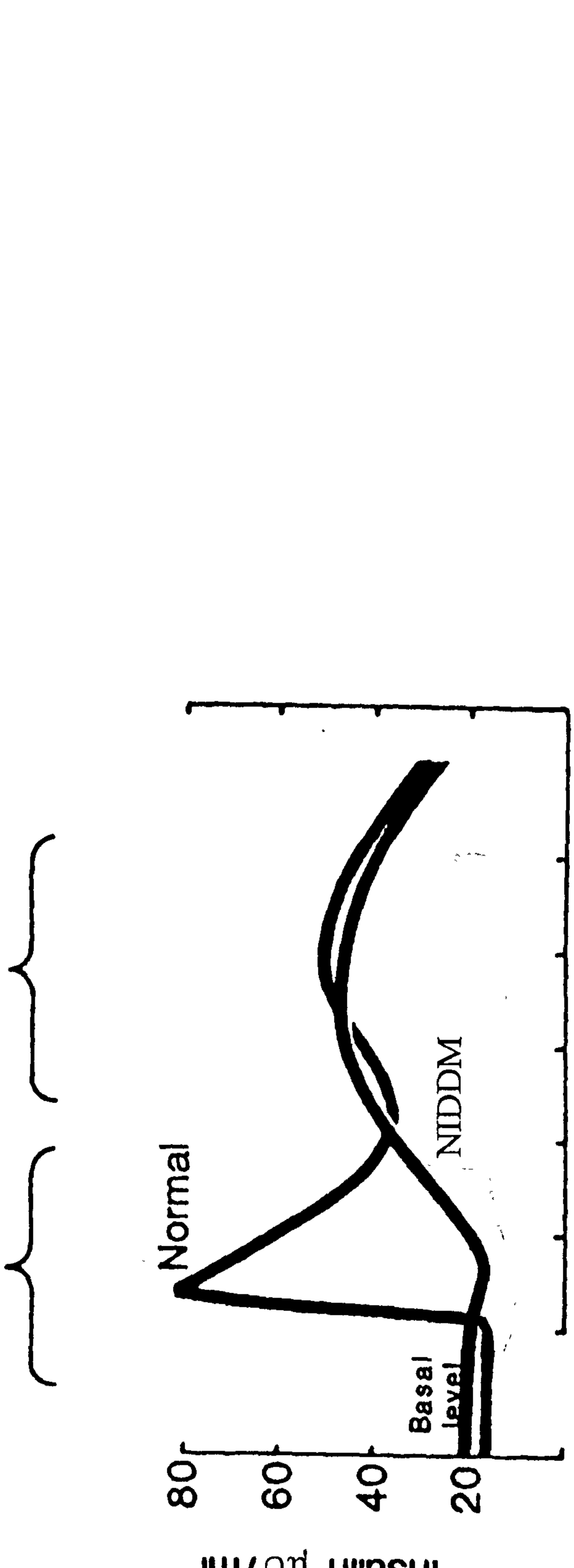


Figure 1.3: Diagram showing the two-phase insulin release during constant glucose infusion
The first phase is missing in NIDDM and both phases are missing in IDDM.

Table 1.1 Summary of the effects of insulin on carbohydrate, fat and protein metabolism in liver, muscle and adipose tissue

	Carbohydrate metabolism	Fat metabolism	Protein metabolism
Liver	↓ gluconeogenesis ↓ glycogenolysis ↑ glycolysis ↑ glycogenesis	↑ lipogenesis ↓ lipolysis	↓ protein breakdown
Adipose tissue	↑ glucose uptake ↑ glycerol synthesis	↑ fatty acid synthesis ↑ synthesis of triglycerides ↓ lipolysis	-----
Muscle	↑ glycogenesis ↑ glycolysis ↑ glucose uptake	-----	↑ amino acid uptake ↑ protein synthesis

Escherichia coli (Johnson, 1983; Martindale, 1996) and yeast. Due to the slight variations in the amino acid sequence of insulin molecules among species, they may be antigenic when administered to a different species. Thus human insulins are particularly chosen in patients who have shown allergic or resistance problems with animal insulins, and for those newly diagnosed IDDM patients.

There are 4 main types of insulin preparations available commercially (Martindale, 1996; BNF, 1998):

- Rapid-acting: e.g. Insulin Lispro
- Short-acting: e.g. Soluble insulin
- Intermediate-acting: e.g. Isophane insulin
- Long-acting: e.g. Insulin Zinc Suspension (Crystalline)

The type of insulin used and its dose and frequency of administration depend on the particular needs of the patient. Most patients are best started on insulins of intermediate action twice daily and a short-acting insulin can be added to cover any hyperglycaemia which may occur after breakfast or evening meal.

Insulin is inactivated by gastrointestinal enzymes, thus it is commonly administered by the subcutaneous route. However, the intravenous route, and rarely, the intramuscular route have also been used for the continuous administration of insulin when more rapid onset of action is required. Other routes of administration of insulin have also been investigated (Chien and Banga, 1989), such as intranasal and rectal administration.

1.1.8 Pancreatic transplantation

Transplantation of the whole pancreas, alone or in combination with a kidney transplant, may be necessary for those IDDM patients whose glycaemic control is poorly controlled by insulin therapy (Remuzzi *et al.*, 1994; Stratta, 1996). However, a more advanced and promising development is the transplantation of pancreatic islets (Morris *et al.*, 1989; Pyzdrowski *et al.*, 1992). A recent study by Secchi *et al.* (1997) showed that islet transplantation has a 45% success rate in terms of insulin-independence or relevant reduction of exogenous insulin requirement, although success can be transient.

1.1.9 Oral hypoglycaemic agents (Table 1.2)

Unlike patients with IDDM who require insulin for survival, all newly diagnosed NIDDM patients are treated with diet alone in the first instance. Drug treatment should be started only when glycaemic control is not achieved after about 3 months trial of dietary modification and increased physical activity. The four major classes of oral hypoglycaemic agents for NIDDM patients are the sulphonylureas, the biguanides, the thiazolidinediones and the α-glucosidase inhibitors.

1.1.9(a) Sulphonylureas

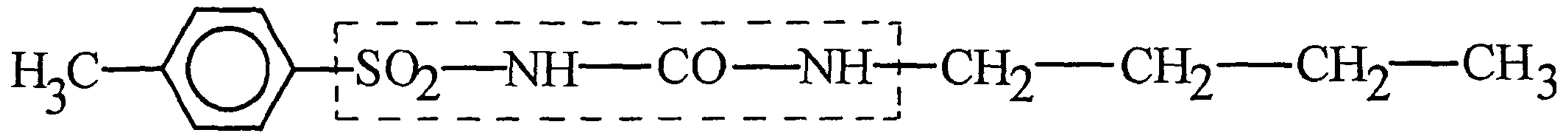
Sulphonylureas are generally used as first line supplement treatment in NIDDM when diet modification has not proved effective on its own. This group of drugs are sulphonamide derivatives; they all possess the sulphonylurea moiety, but different substitutions (Fig. 1.4) result in differences in pharmacokinetics and side effect profile. Sulphonylureas (Grodsky *et al.*, 1977) act mainly by stimulating insulin secretion and consequently are effective only when some residual pancreatic β-cell activity is present (about 30% of normal β-cell function). The site of action of sulphonylureas is the ATP-sensitive potassium channel in the membrane of β-cells (Sturgess *et al.*, 1985). In addition, they inhibit hepatic glucose production and reduce peripheral resistance to insulin action (Gale, 1990). Several sulphonylureas are available but there is no evidence for any difference in their effectiveness. However, the duration of action of sulphonylureas is variable; drugs such as tolbutamide and gliclazide are relatively short-acting (approximately 8 and 12 hours respectively), while chlorpropamide has a prolonged action of 48 hours.

Sulphonylureas are usually well tolerated. Hypoglycaemia is the most common side effect and its incidence is related to the potency and duration of action of the drug. Skin rashes, gastrointestinal disturbances and headache may also occur (Paice *et al.*, 1985). In addition, sulphonylureas stimulate appetite and thus cause weight gain which is the major concern in obese NIDDM patients. However, unlike other sulphonylureas, chlorpropamide has a wider range of side effects (facial flushing after alcohol intake, enhanced antidiuretic hormone and very rarely hyponatraemia) and is now less widely prescribed.

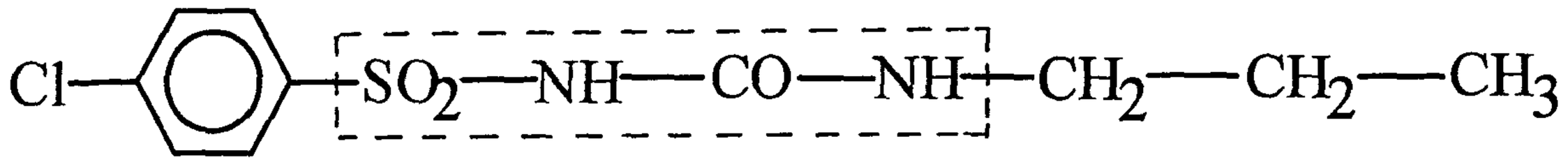
Table 1.2 Classification of oral hypoglycaemic agents

Different classes	Examples Active ingredients	Proprietary preparations (Martindale,1996; BNF, 1998)
Sulphonylureas	Chlorpropamide	Diabinese®
	Glibenclamide	Daonil®, Semi-Daonil®, Euglucon®
	Gliclazide	Diamicron®
	Glipizide	Glibenese®, Minodiab®
	Gliquidone	Glurenorm®
	Tolazamide	Tolanase®
	Tolbutamide	Rastinon®
Biguanides	Metformin	Glucophage®
	Phenformin	Gliformin® (Italy), Diabis Activado® (Spain)
Thiazolidinediones	Troglitazone	Romozin® **
	Pioglitazone	----
Alpha glucosidase inhibitors	Acarbose	Glucobay®
	Emiglitate	-----
	Miglitol	-----
Aldose reductase inhibitors	Epalrestat	Kinedak® (Japan)
	Tolrestat	Alredase® (Eire, Italy)
Miscellaneous	Guar Gum	Guarem®

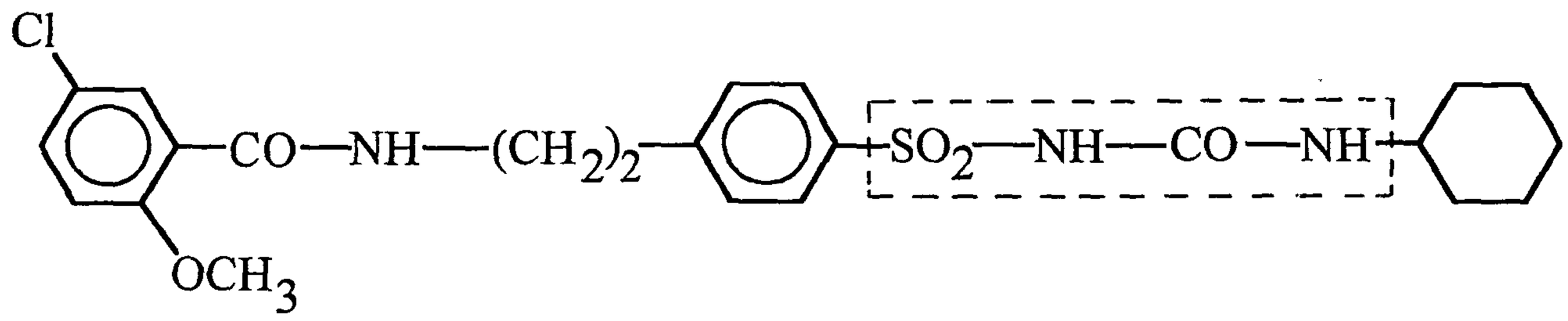
** Withdrawn from use in UK in December 1997



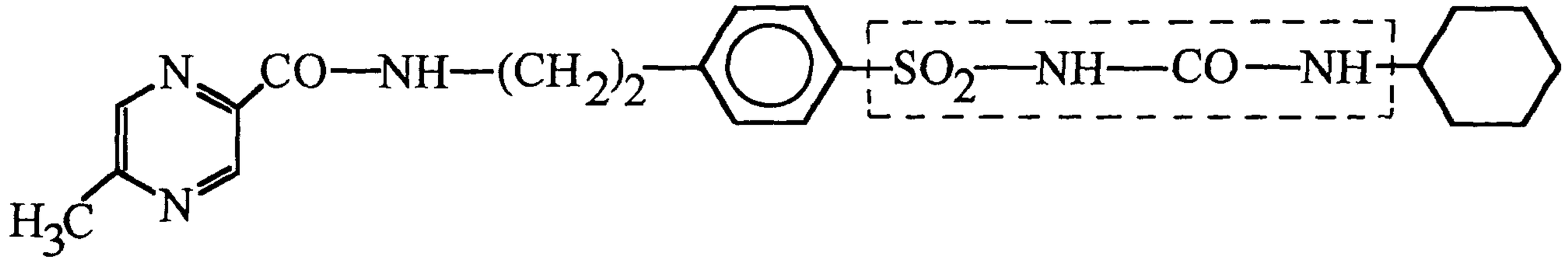
Tolbutamide



Chlorpropamide



Glibenclamide



Glipizide

Figure 1.4: Structures of some sulphonylurea drugs
(The sulphonylurea moiety is shown within the dotted area)

The main selection factors for a particular sulphonylurea include the age of the patient (the long-acting drugs such as chlorpropamide and glibenclamide should be avoided in elderly patients who are particularly prone to the danger of hypoglycaemia) and renal function (the short-acting tolbutamide may be used in renal impairment, as well as gliquidone and gliclazide which are metabolised in the liver).

Many compounds have been reported to interact with sulphonylureas (Martindale, 1996). Drugs that increase the hypoglycaemic effect of sulphonylureas include non-steroidal anti-inflammatory agents, monoamine oxidase inhibitors, some antibacterials, antifungals and some uricosuric drugs. The interaction is due to competition for metabolising enzymes, and interference with plasma protein binding since all sulphonylureas bind strongly to plasma albumin. Some compounds such as rifampicin, thiazide diuretics, and corticosteroids diminish the hypoglycaemic effect of sulphonylureas.

1.1.9(b) Biguanides

Metformin (Fig. 1.5), the only biguanide available in the UK, is used in the treatment of NIDDM when strict dieting and sulphonylurea treatment have failed to control diabetes. Metformin can also be used as initial therapy, especially in obese NIDDM patients since it is not associated with weight gain. It can be used alone or in combination with the sulphonylureas.

Metformin (Perriello, 1995; Bailey and Turner, 1996) has a different mode of action from the sulphonylureas. It exerts its effect mainly by inhibiting hepatic gluconeogenesis and by increasing peripheral utilisation of glucose. Metformin therapy also improves insulin sensitivity; since it only acts in the presence of endogenous insulin, it is only effective in diabetic patients with some residual functioning pancreatic islet cells. It may also act by delaying glucose absorption from the gastrointestinal tract, although overall absorption of a glucose load is not reduced. In addition, metformin decreases fatty-acid oxidation, this results in a reduction in plasma glucose concentration by means of the glucose-fatty-acid cycle.

Metformin is absorbed from the gastrointestinal tract, mainly from the small intestine. It has a plasma half-life of about 3 hours and is excreted unchanged in the urine.

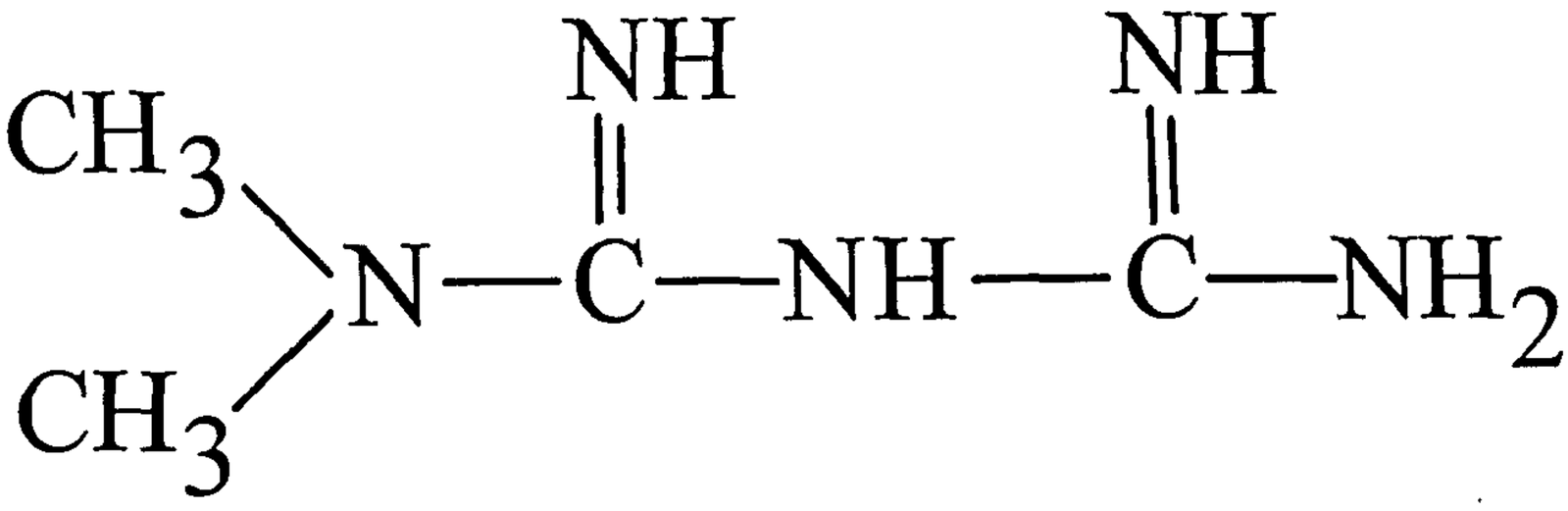


Figure 1.5: Structure of Metformin

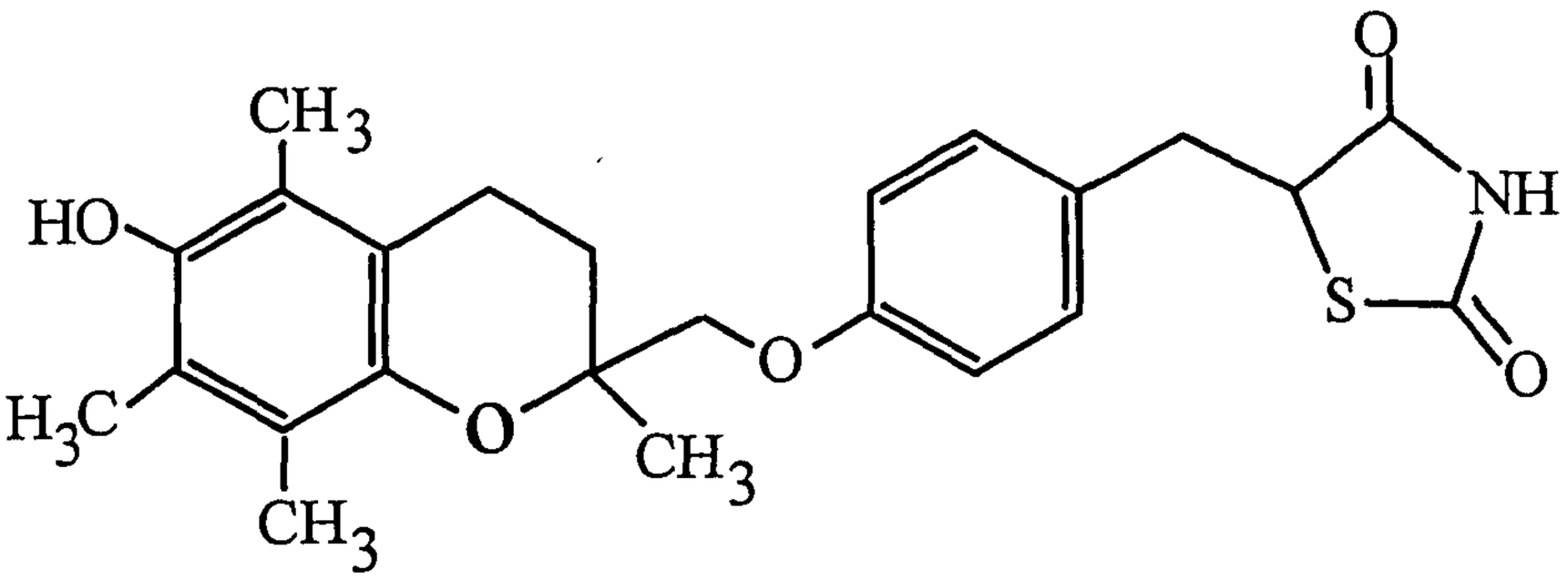


Figure 1.6: Structure of Troglitazone

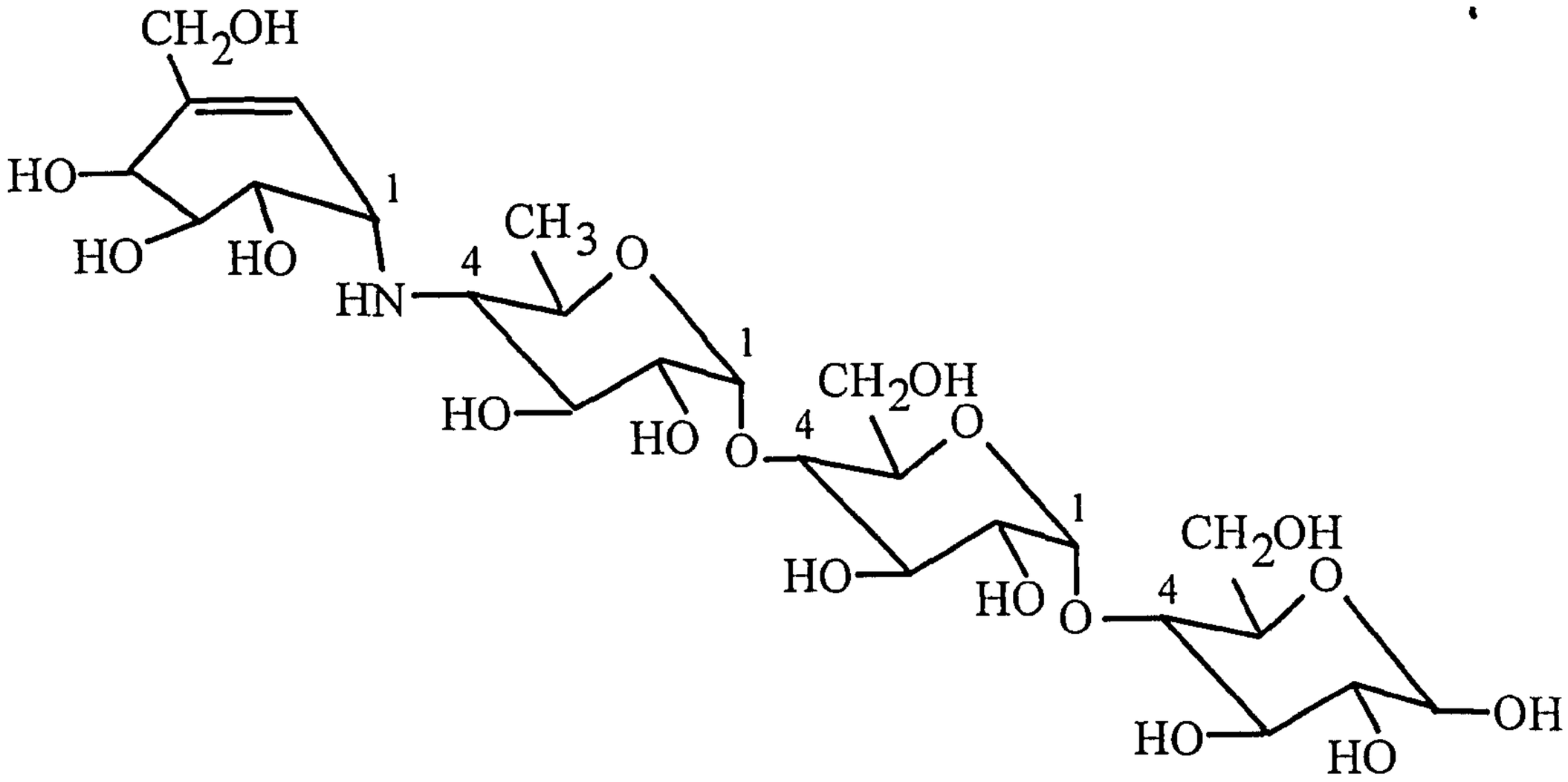


Figure 1.7: Structural formula of Acarbose ($C_{25}H_{43}O_{18}N$)

Unlike sulphonylureas, metformin is not bound to plasma proteins and thus results in fewer drug interactions. The main drug interaction is with cimetidine (ulcer-healing drug) which inhibits renal tubular secretion of metformin and thus increases plasma metformin concentrations (Somogyi *et al.*, 1987). Intestinal absorption of vitamin B_{12} and folic acid may be impaired during long-term treatment (Bailey, 1992).

Metformin has the advantages of low incidence of weight gain and lower plasma insulin levels. It does not induce hypoglycaemia in normal subjects. Lactic acidosis is a rare but serious adverse effect in metformin-treated patients; it occurs almost exclusively in patients with renal impairment in whom the use of metformin is contra-indicated. Other side effects include anorexia, nausea, vomiting and diarrhoea. Patients may experience a metallic taste and there may be weight loss.

1.1.9(c) Thiazolidinediones

Thiazolidinediones belong to a new class of oral hypoglycaemic agents. They are potent and specific activators of peroxisome proliferator-activated receptor γ (PPAR γ), a member of the ligand activated nuclear receptor subfamily. There is a striking correlation between the clinical activity of the various thiazolidinediones and their ability to activate PPAR γ (Grossman and Lessem, 1997). PPAR γ is expressed more specifically in adipose tissue and is implicated in adipogenesis. In cell culture models, thiazolidinediones act as potent adipogenic agents reflecting their ability to activate PPAR γ (Wilson *et al.*, 1996). These observations led to the suggestion that the anti-diabetic actions of thiazolidinediones result from their ability to alter adipocyte differentiation and/or metabolism resulting in lower serum lipid levels and to possibly decrease the secretion of humoral factors from adipocytes, such as TNF-α, which would result in the improvement of insulin sensitivity (Grossman and Lessem, 1997).

Troglitazone (Fig. 1.6) is the first thiazolidinedione which was very recently launched in UK as a new treatment for diabetes (Anonymous, 1997a). It was indicated for the treatment of NIDDM, either as monotherapy in patients inadequately controlled by diet, or in combination with sulphonylureas or insulin to improve glycaemic control. In clinical studies, troglitazone has been shown to decrease insulin resistance (enhances insulin action), reduce hyperinsulinaemia, reduce hepatic glucose

production and improve both fasting and postprandial glycaemia in NIDDM patients (Iwamoto *et al.*, 1991; Suter *et al.*, 1992; Nolan *et al.*, 1994). It is hoped that troglitazone will reduce both microvascular complications of NIDDM (e.g. retinopathy and nephropathy) by its effects on glucose control, and macrovascular complications (e.g. myocardial infarction and stroke) by its effects on insulin and lipid levels, though the long term effect on reduction of complications is yet to be investigated.

Troglitazone monotherapy does not induce hypoglycaemia. However, it may potentiate the hypoglycaemic effects of insulin and sulphonylureas and a reduction in dosage of one agent may be necessary. Reported side effects include diarrhoea, nausea, vomiting, headache, malaise and musculoskeletal pain. It is contraindicated in patients with severe hepatic impairment since 40 cases of serious hepatic dysfunction (severe hepatocellular damage, hepatic necrosis and hepatic failure) occurring during treatment with troglitazone have been reported worldwide since its introduction (Anonymous, 1997b). At present, no clear risk factors for the development of hepatic reactions have been identified which might allow the drug to be used safely in some patients. Since the risks of troglitazone therapy outweigh the potential benefits, troglitazone had been withdrawn in the UK from December 1997 (Anonymous, 1997c), though it remains available in Japan and in the United States.

Despite the serious hepatic adverse effects associated with troglitazone, the thiazolidinedione class of anti-hyperglycaemic agents may still be the most significant advance in the treatment of NIDDM since the advent of the sulphonylureas and biguanides in the 1950s. Other thiazolidinediones which are currently under clinical development include pioglitazone and BRL 49653 (Turner, 1996). The fact that whether the hepatic problems are a class effect remains to be answered.

1.1.9(d) Alpha-glucosidase inhibitors

In recent years, the alpha-glucosidase inhibitor **acarbose** has also been used in the treatment of diabetes. Acarbose, produced by fermentation of strains of *Actinoplanes*, is a pseudotetrasaccharide in which one maltose residue is replaced by a pseudomaltose residue, which is essential for the inhibitory effect of the compound

(Fig. 1.7). Acarbose acts in the intestine by inhibiting intestinal α-glucosidases located within the upper intestinal brush border. This results in delaying the digestion of carbohydrates and postponing the absorption of glucose from disaccharides, oligosaccharides and polysaccharides in the small intestine (Caspary and Graf, 1979), and hence reduces postprandial rises of blood glucose and smoothes out the 24-hour blood glucose profiles. Acarbose, given alone or combined with other oral hypoglycaemic agents, is an effective treatment for NIDDM (Chiasson *et al*., 1994); it can also be used to supplement insulin therapy in IDDM which improves glycaemic control and decreases insulin requirement.

Following oral administration of acarbose, less than 2% is absorbed in its active form; the majority of active unchanged drug remains in the lumen of the gut to exert its pharmacological activity. It is metabolised by intestinal enzymes and by the microbial flora and is excreted in the urine and faeces.

Acarbose may cause gastrointestinal disturbances, including flatulence due to bacterial action on non-absorbed carbohydrate in the colon. Bowel sounds, abdominal distension, diarrhoea and pain may also occur. Acarbose may potentiate the effects of other hypoglycaemic drugs and insulin, thus reduction in their dosage may be required.

Other α-glucosidase inhibitors such as Emiglitate and Miglitol, which have similar actions as acarbose, are still under investigation for their use in the management of diabetes mellitus (Martindale, 1996).

1.1.9(e) Other agents used in the treatment of diabetes or its complications

Guar gum is used as an adjunct to treatment with diet, insulin, or other oral hypoglycaemics in the management of diabetes mellitus. Guar gum is obtained from the ground endosperms of the seeds of *Cyamopsis tetragonoloba* (Leguminosae) and is a high molecular weight hydrocolloidal polysaccharide composed of galactan and mannan units connected by glycosidic linkages.

Guar gum reduces postprandial and fasting plasma glucose concentrations as well as plasma insulin concentrations in diabetic patients (Blackburn *et al.*, 1984; Hockaday, 1990). The possible modes of action include delayed gastric emptying, decreased small bowel motility, decreased glucose absorption as a result of increased viscosity of the contents of the gastrointestinal tract, and inhibition of gastrointestinal hormones.

Like acarbose, guar gum can cause gastrointestinal disturbance with flatulence, abdominal distension, diarrhoea and nausea. Its use is contraindicated in patients with gastrointestinal obstruction. Guar gum may also affect the absorption of other drugs such as phenoxymethylpenicillin.

Aldose reductase inhibitors such as Epalrestat and Tolrestat inhibit the enzyme aldose reductase which catalyses the conversion of glucose to sorbitol. It has been found that accumulation of sorbitol in certain cells only occurs in hyperglycaemic conditions and results in a hyperosmotic effect, which may be involved in the pathogenesis of some diabetic complications. However, aldose reductase inhibitors have no influence on plasma glucose concentrations. Both Tolrestat and Epalrestat are used in the treatment of diabetic neuropathy (Masson and Boulton, 1990). The use of aldose reductase inhibitors in the treatment of other diabetic complications such as diabetic retinopathy and nephropathy is still under investigation.

1.1.9(f) Potential new anti-diabetic agents

Glucagon-like peptide 1 (GLP-1), a 37 amino acid peptide, is a gastrointestinal hormone capable of enhancing glucose-stimulated insulin release by β-cells (Nauck *et al.*, 1993) and decreasing blood glucose in NIDDM patients (Amiel, 1994). Because of the glucose-dependent effect, it is associated with a low risk of hypoglycaemia. However, its ability to inhibit gastric emptying leads to gastrointestinal discomfort.

Repaglinide is a new non-sulphonylurea, insulin-stimulating drug. It appears to stimulate postprandial insulin response and its blood glucose-lowering effect seems to be similar to that of the sulphonylureas (Wolfenbuttel *et al.*, 1993; Balfour and Faulds, 1998). It stimulates insulin secretion by blocking the ATP-dependent

potassium channel. Other non-sulphonylurea secretogogues include A4166, NN623 and KAD-1229 (Ohnota *et al.*, 1995; Kikuchi, 1996).

Mecasermin (insulin-like growth factor I) has been shown to improve metabolic control in patients with extreme insulin resistance and improve glucose tolerance and decrease hyperinsulinaemia in NIDDM patients. (Kolaczynski and Caro, 1994).

Midaglizole is under investigation as a hypoglycaemic agent. It is believed to stimulate insulin secretion by antagonism of α_2-adrenoceptors (Kawazu *et al.*, 1987).

Other approaches under investigation include inhibition of fatty acid oxidation or the use of **β_3-adrenoceptor agonists** (Turner, 1996). The potent anti-obesity and anti-diabetic properties of agonists at β_3-adrenoceptors in rodents has led to interest in their potential as agents for the treatment of obesity and diabetes (Arch and Kaumann, 1993; Blin *et al.*, 1993). In animal studies (Largis *et al.*, 1994), β_3-agonists elicited a reduction in weight gain in obese rodents without a concomitant decrease in food intake, suggesting that it was a consequence of increased thermogenesis. Thus chronic treatment of obese animals with β_3-agonists causes a sustained increase in metabolic rate, body temperature and 24h energy expenditure. The reduction in body weight is due to a reduction in body lipid content, consistent with the potent lipolytic activity of β_3-agonists. β_3-Agonists also improve glycaemic control and insulin sensitivity in NIDDM animals. FR149175, a β_3-agonist was shown to improve hyperinsulinaemia and glucose tolerance in NIDDM obese rats (Yamamoto *et al.*, 1997). β_3-agonists are thought to interact with elements of the insulin signal transduction cascade in order to improve the sensitivity of target tissues to insulin (Rochet *et al.*, 1988; Le Marchand-Brustel *et al.*, 1990). However, their efficacy in man is doubtful due to species-specific differences between rodent and human receptors (Strosberg, 1997). Thus the effect of β_3-agonists in man requires further investigations.

Patients with NIDDM who cannot be controlled adequately by oral therapy and diet need to be given insulin either in addition to the existing treatment or in place of the oral therapy.

1.1.10 Use of plants in the treatment of diabetes

Apart from the conventional drug therapy, it is important to mention that some plants have also been used as traditional remedy for the treatment of diabetes. Before the advent of insulin and oral hypoglycaemic drugs, plant medicines represented the predominant treatment for diabetes mellitus. Due to the inability of current anti-diabetic therapies to control all of the pathological aspects of diabetes, as well as the high cost and poor availability of current drug treatments for many rural populations, especially in developing countries, alternative drugs are clearly needed. Since people in rural areas of developing countries have long been and still are relying on traditional medicines for their primary health care, a scientific investigation of traditional herbal remedies for diabetes may provide valuable leads for the development of new anti-diabetic drugs. Such studies may lead to validation of these remedies and provide the basis for the development of standardised therapies for use in poorer countries. In fact, metformin (one of the most prescribed oral glucose-lowering drug) is derived from a medicinal plant, *Galega officinalis*, historically used to treat diabetes (Bailey and Day, 1989). More than 400 different plants with possible glucose-lowering actions are known and some of them will be discussed in detail in the following sections.

1.2 Anti-diabetic plants and their active constituents

In recent years, there has been a renewed interest in plant-derived pharmaceuticals in the Western world. This is demonstrated not only by the increasing number of active research programmes into natural products conducted by major pharmaceutical companies worldwide, but also the general public who are increasingly using plant extracts or herbal medicine as opposed to conventional medicines in self medication. In fact, the trade in herbal remedies in the European Union in 1990 was valued at over $1000 million and rising at 13% per year (McAlpine, 1992).

Plants are undoubtedly a potential source for new drugs or lead compounds for drug development (Farnsworth, 1994). In the past, many important drugs have been derived from natural sources and still remain the drugs of choice for particular medical conditions. Examples are the vinca alkaloids, vinblastine and vincristine, of *Catharanthus roseus* (Apocynaceae) used in cancer chemotherapy, the cardiac glycoside digoxin from *Digitalis lanata* (Scrophulariaceae), the analgesics morphine and codeine obtained from the Opium poppy *Papaver somniferum* (Papaveraceae), and more recently, the diterpene taxol from the Pacific yew *Taxus brevifolia* (Taxaceae) used for treating ovarian and breast cancer.

As with other therapeutic areas, many plants have been used for the treatment of diabetes mellitus in traditional systems of medicine throughout the world. A number of review articles have been published on the traditional use of plants in diabetes (Bailey and Day, 1989; Swanston-Flatt *et al.*, 1991) and on plants and phytochemicals whose reputed hypoglycaemic effects have been scientifically investigated (Perl, 1988; Handa *et al.*, 1989; Day, 1990; Perez G. *et al.*, 1998). The most extensive review is by Marles and Farnsworth (1995) who evaluated available data on more than 1200 species of plants reported either to have been used to treat diabetes and/or to have been investigated for anti-diabetic activity. The review indicated that more than 80% of those traditional plant remedies studied pharmacologically were demonstrated to have hypoglycaemic activity, resulting from a great variety of mechanisms of action. The aim of this section is to look at some plants which have been used traditionally for the treatment of diabetes mellitus and to present some natural compounds which possess anti-diabetic activity. These may

have the potential to act as lead compounds for the development of new anti-diabetic treatments.

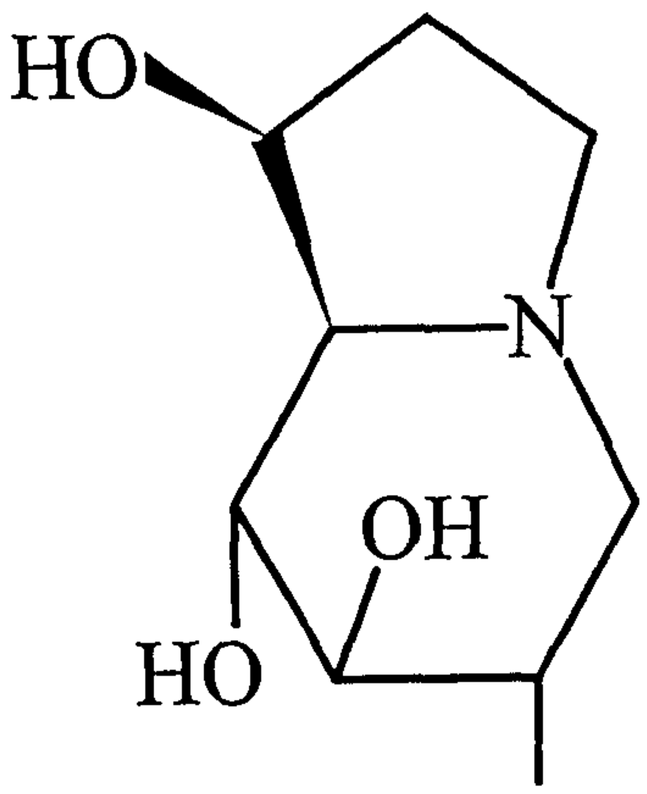
In this context, the discovery process (Oubré *et al.*, 1997) that led to the use of metformin cannot be omitted. *Galega officinalis* L. (Fabaceae), also known as goat's rue or French lilac, was used as a treatment for diabetes in medieval Europe (Bailey and Day, 1989). Guanidine, the main component in *Galega officinalis*, was shown to produce hypoglycaemia in parathyroidectomized test animals by parenteral administration. Since this compound was too toxic for clinical use, alkyl diguanides were synthesized from it in the 1920s. Biguanidines (e.g. metformin and phenformin), a third generation of guanidine derivatives, were introduced in 1957 as a new class of anti-diabetic agents. To date, metformin remains an efficacious drug approved for treatment of NIDDM derived from a medicinal plant historically used to treat diabetes (Section 1.1.9(b)).

A number of mechanisms by which phytochemicals may exert their hypoglycaemic activity have been described by Perl (1988). Thus herbal remedies for diabetes can be classified into different categories according to their mode of action as follows:

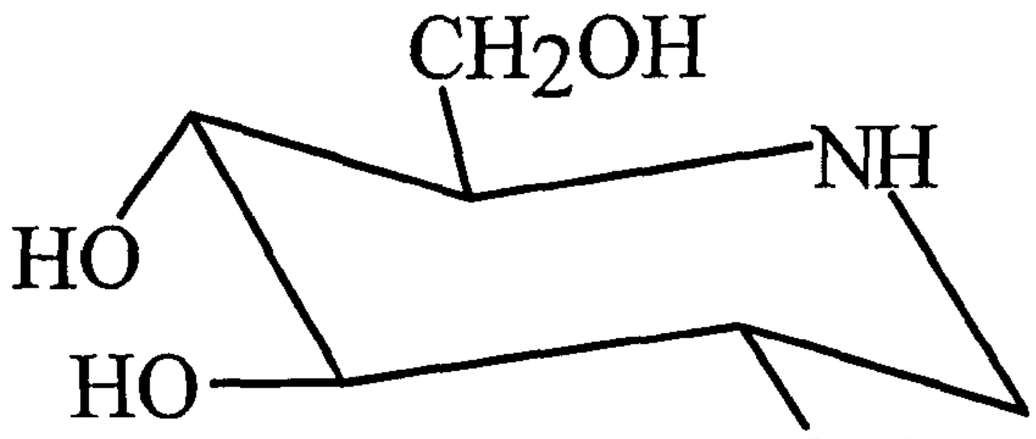
1.2.1 Inhibition of glucose absorption from the gastrointestinal tract

Cyamopsis tetragonolobus* Taub. (Fabaceae).** Guar gum (Section 1.1.9(e)) is a purified form of dietary fibre extracted from the seeds of Indian cluster bean *C. tetragonolobus*. It contains not less than 66% of high molecular weight hydrocolloidal polysaccharide galactomannan, which is known to reduce post-prandial hyperglycaemia (Blackburn *et al.*, 1984; Day, 1990). Guar gum inhibits intestinal absorption of glucose by slowing gastric emptying and by thickening the unstirred water layer adjacent to the intestinal villi which acts as a barrier to diffusion (Hakim *et al.*, 1995). Other traditional remedies which may work in this way include acacia gum (Acacia senegal* Wild.**) and some other species of *Acacia* of the Fabaceae family (Marles and Farnsworth, 1995).

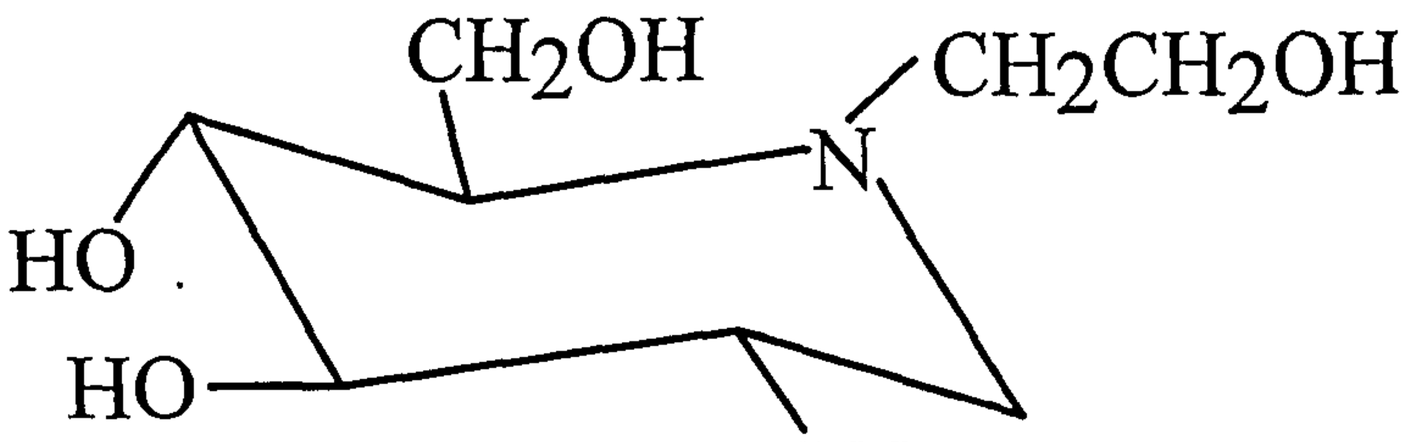
***Castanospermum australe* A. Cunn. (Fabaceae).** Castanospermine (Fig. 1.8), an indolizidine alkaloid isolated from *C. australe*, is an intestinal enzyme (α-glucosidase) inhibitor with hypoglycaemic activity. It blocks the hyperglycaemic



Castanospermine



Moranoline



Miglitol

Figure 1.8: Hypoglycaemic intestinal enzyme inhibitors from plants

response to oral doses of sucrose through inhibition of disaccharidase, but does not improve oral glucose tolerance. (Rhinehart *et al.*, 1987).

***Morus alba* L. (Moraceae).** Moranoline (Fig. 1.8), isolated from mulberry root bark is a potent intestinal α-glucosidase inhibitor (Yoshikuni, 1988). Miglitol (Fig. 1.8), a semi-synthetic derivative of moranoline, is also an α-glucosidase inhibitor which, unlike acarbose (Section 1.1.9(d)), is well absorbed from the gut.

Phlorizin/phloridzin (Fig. 1.9), a phenolic glycoside present in plants of the Rosaceae family, especially species of the genus ***Malus***, is a Na^+-dependent sugar transport inhibitor (Schultz and Curran, 1970) which prevents glucose absorption from the gut and inhibits glucose reabsorption by the kidneys.

1.2.2 Modification of insulin levels

***Pterocarpus marsupium* Roxb. (Papilionaceae).** The flavonoid, epicatechin (Fig. 1.10), which is the active principle isolated from the heartwood of *P. marsupium*, has been shown to cause an ATP-dependent enhancement of glucose-stimulated insulin secretion from isolated pancreatic islets *in vitro*, and to cause a rise in islet insulin content *in vivo* in rats (Marles and Farnsworth, 1995).

***Allium cepa* L.** and ***A. sativum* L. (Liliaceae).** The hypoglycaemic principles of onion (*A. cepa*) and garlic (*A. sativum*) are the sulphur-containing compounds, allyl propyl disulphide and diallyl disulphide oxide, allicin (Fig. 1.11). Instead of direct stimulation of insulin release, these compounds are believed to act by competing with insulin, which has a disulphide linkage, for endogenous sulphydryl-rich insulin-inactivating compounds (Augusti *et* al., 1974; Marles and Farnsworth, 1995).

Other anti-diabetic plants which may act on insulin secretion include ***Aloe arborescens* Mill. (Liliaceae), *Bridelia ferruginea* Benth. (Euphorbiaceae), *Clerodendron phlomidis* Linn. (Verbenaceae), *Ficus bengalensis* Linn. (Moraceae)** and ***Poterium ancisroides* Desf. (Rosaceae)** (Handa *et al.*, 1989; Hakim *et al.*, 1995).

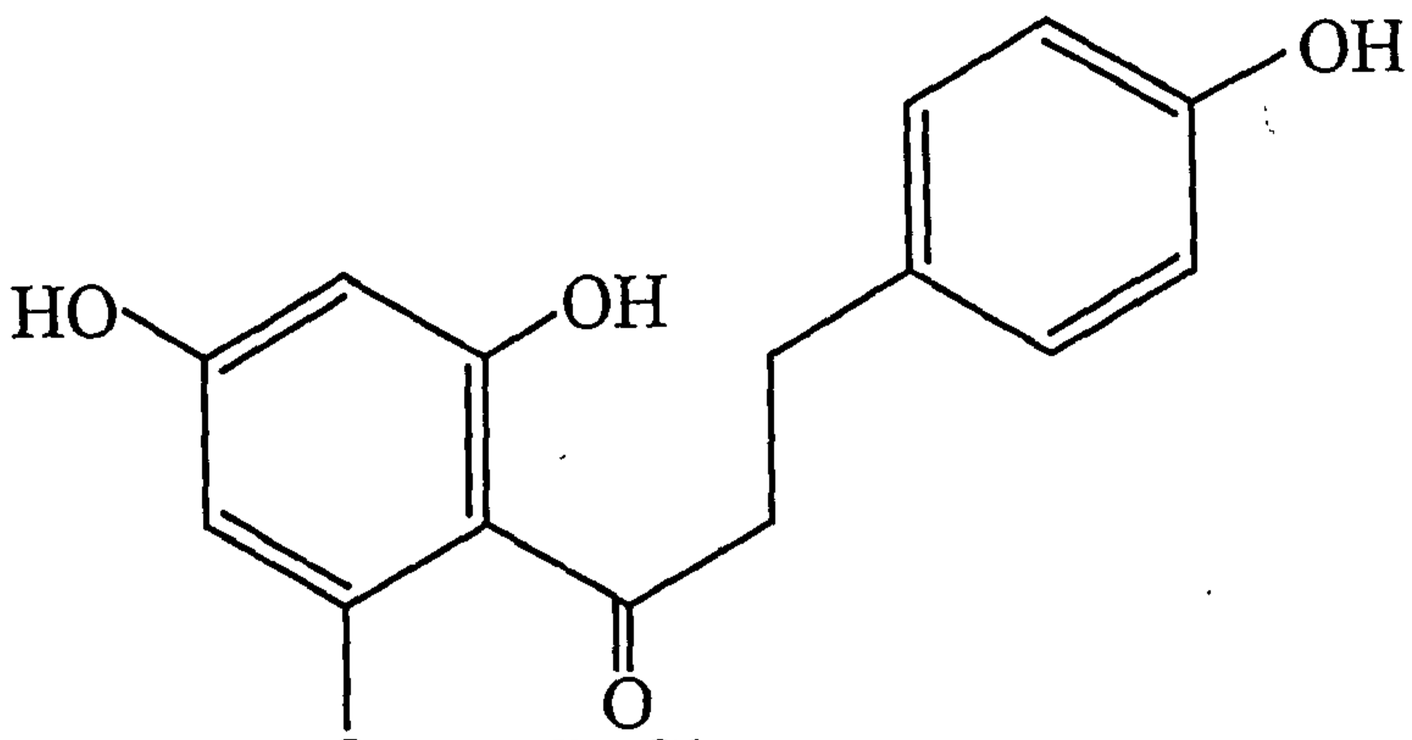


Figure 1.9: Phlorizin

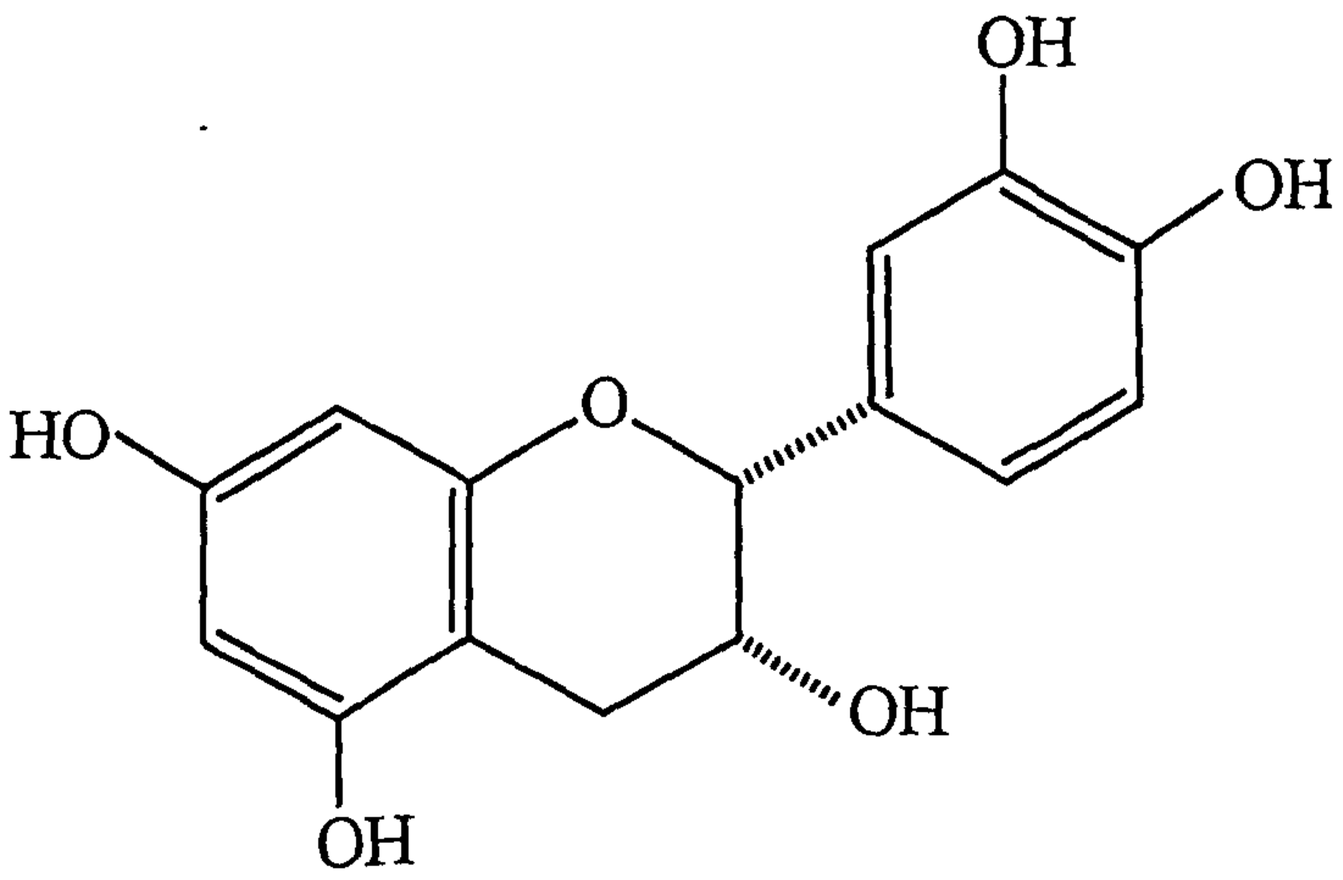


Figure 1.10: Epicatechin, isolated from *Pterocarpus marsupium*

$CH_2=CH-CH_2-S-S-CH_2CH_2CH_3$

Allyl propyl disulphide

$CH_2=CH-CH_2-S(\rightarrow O)-S-CH_2-CH=CH_2$

Diallyl disulphide oxide

Figure 1.11: Hypoglycaemic sulphur-containing compounds present in *Allium* species

1.2.3 Increased glucose utilisation

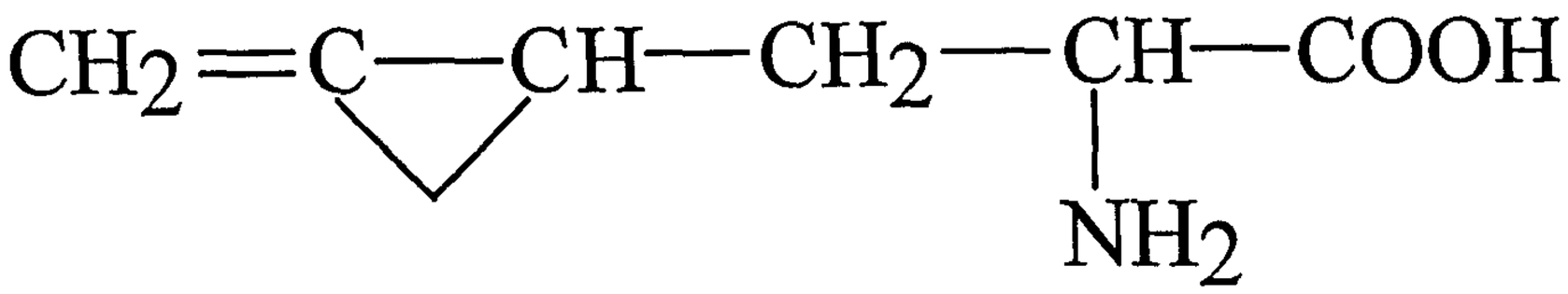
***Blighia sapida* Koen. (Sapindaceae).** Two potent hypoglycaemic agents have been isolated from the unripe fruits of the Jamaican akee tree, *B. sapida*. They are cyclopropanoid amino acids known as hypoglycin A and its γ-L-glutamyl dipeptide, hypoglycin B (Fig. 1.12). They act by inhibiting β-oxidase enzymes, thus blocking the pathway of oxidation of long-chain fatty acids. Since fatty acids are no longer available as energy source, hepatic glycolysis is stimulated to provide an alternate energy source, and the enhanced glucose utilisation results in a fall in blood glucose levels. However, these hypoglycins are too toxic to be used therapeutically (Handa *et al.*, 1989; Marles and Farnsworth, 1995).

***Galega officinalis* L. (Fabaceae).** Seeds of the traditional anti-diabetic plant, *G. officinalis*, contain a high concentration of galegine (isoamylene guanidine, Fig. 1.13). Galegine blocks succinic dehydrogenase, thereby inhibiting the Kreb's cycle and increasing glucose utilisation by anaerobic respiration which leads to hypoglycaemia (Perl, 1988).

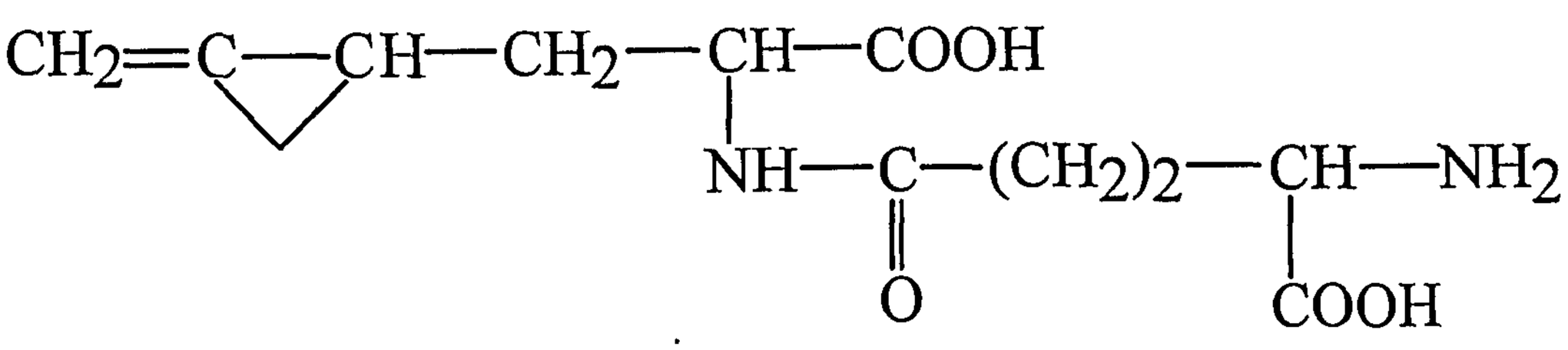
1.2.4 Other plants with hypoglycaemic activity due to various modes of action

Many of the plants discussed above are reported to contain a single hypoglycaemic component which appears to exert the hypoglycaemic activities *via* a particular mode of action. However, some anti-diabetic plants have been shown to contain more than one active constituent which may act *via* different mechanisms of action:

***Gymnema sylvestre* R.Br. (Asclepiadaceae).** *Gymnema sylvestre*, also known as "gurmar" due to its property of temporarily abolishing the appreciation of sweet taste by the tongue after chewing the leaves, is a traditional Ayurvedic remedy for the treatment of diabetes. *G. sylvestre* leaf extract was shown to increase plasma insulin concentrations and raise levels of insulin-sensitive enzymes such as hexokinase in diabetic animal models (Shanmugasundaram *et al.*, 1983). An ethanolic extract of *G. sylvestre* containing gymnemic acids and named "GS4" was found to decrease fasting plasma glucose levels and increase plasma insulin levels in NIDDM patients (Baskaran *et al.*, 1990); and decrease insulin requirements in IDDM patients



Hypoglycin A



Hypoglycin B

Figure 1.12: Inhibitors of fatty acid oxidation isolated from *B. sapida*

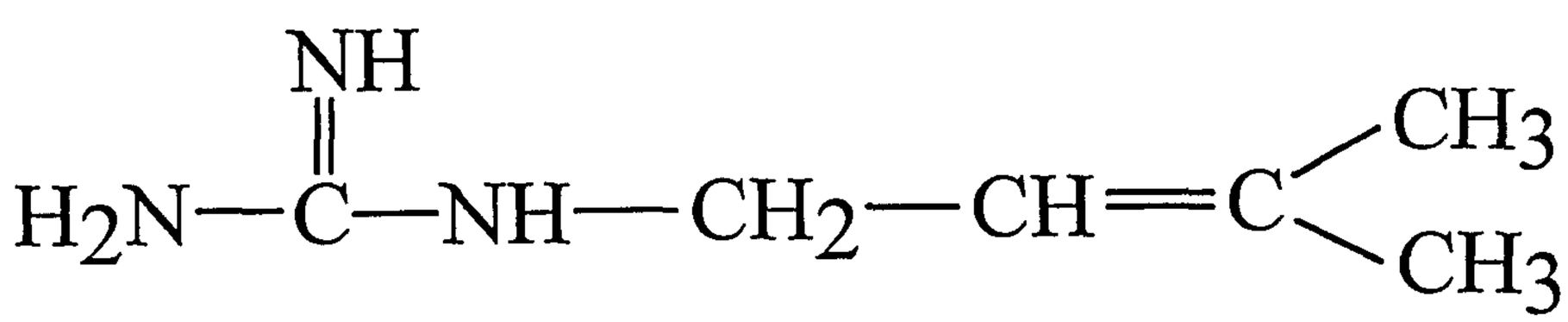


Figure 1.13: The guanidine derivative, galegine, found in *G. officinalis*

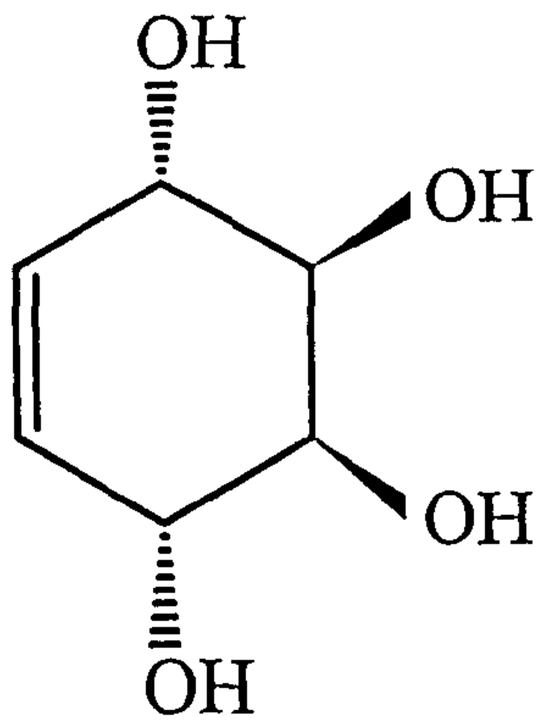
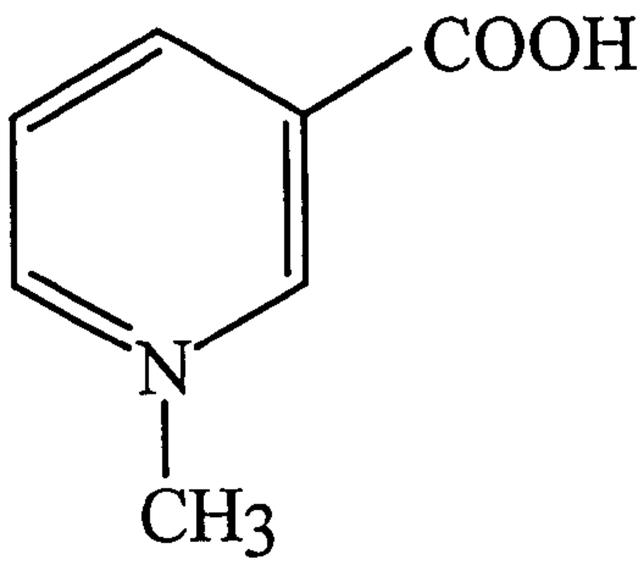


Figure 1.14: Conduritol A, isolated from *Gymnema sylvestre*

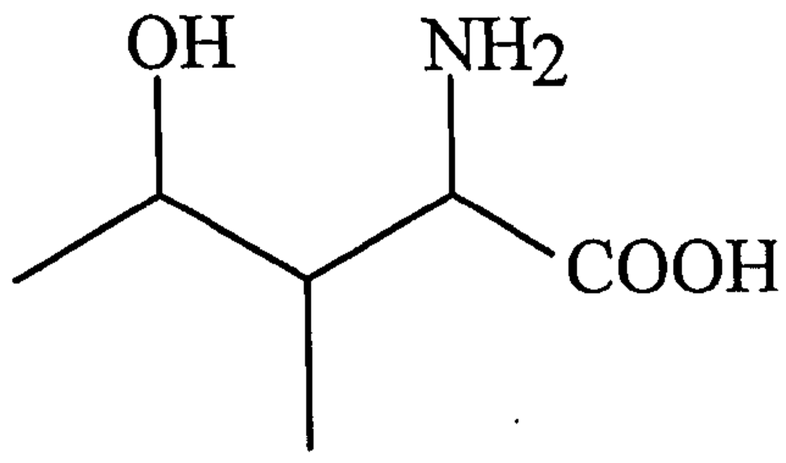
(Shanmugasundaram *et al.*, 1990).

A compound chemically unrelated to gymnemic acids, conduritol A (Fig. 1.14), isolated from *G. sylvestre* was also shown to modulate insulin secretion from isolated pancreatic islets (Billington *et al.*, 1994). On the other hand, both conduritol A and the triterpenoid glycosides, gymnemic acids (also present in *G. sylvestre*) have been reported to inhibit intestinal glucose absorption (Yoshioka, 1986; Miyatake *et al.*, 1993). Thus the plant *G. sylvestre* may exhibit its hypoglycaemic effect by stimulating insulin secretion as well as inhibiting intestinal glucose absorption.

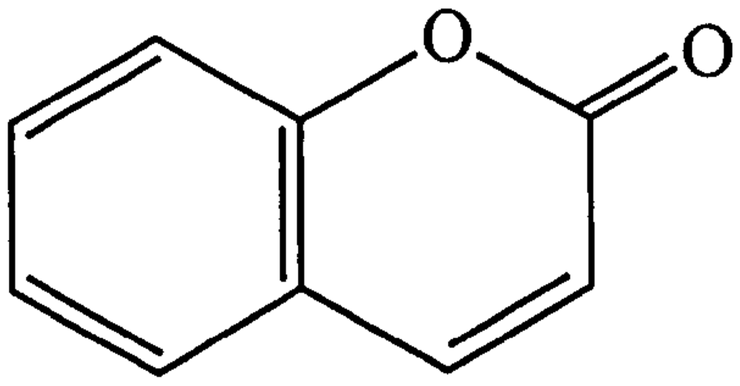
Trigonella foenum-graecum **Linn. (Papilionaceae).** Fenugreek (*T. foenum-graecum)* seeds have long been used as a herbal remedy for the treatment of diabetes. The seeds are known to contain a number of hypoglycaemic principles. Early reports suggested that the hypoglycaemic effect of fenugreek was attributed to its major alkaloid, trigonelline (Fig. 1.15), the N-methyl derivative and main human metabolite of the vitamin nicotinic acid (Mishkinsky *et al.*, 1967). However, administration of trigonelline (at a dose equivalent to that present in fenugreek) to diabetic patients did not show any significant hypoglycaemic activity (National Institute of Nutrition, 1987). Hillaire-Buys *et al.* (1993) reported the presence of an insulin-stimulating substance in the seeds of fenugreek, identified as 4-hydroxyisoleucine (Fig. 1.15). Other proposed hypoglycaemic constituents of fenugreek are coumarin (Fig. 1.15), which was shown to have significant hypoglycaemic effect in normal and alloxan-induced diabetic rats (Shani *et al.*, 1974) and scopoletin (Fig. 1.15), another coumarin constituent of fenugreek, which exerted borderline hypoglycaemic effects in normal and alloxan-induced diabetic rats at high doses (Shani *et al.*, 1974). Ghosal *et al.* (1974) reported that a steroidal sapogenin-peptide ester, fenugreekine (Fig. 1.15) also possessed hypoglycaemic effect. In addition, Ali *et al.* (1995) showed that the soluble dietary fibre fraction of fenugreek seed (containing galactomannan as the major constituent) had a significant anti-hyperglycaemic effect but not hypoglycaemic effect in NIDDM model rats, suggesting an action at the intestinal level.



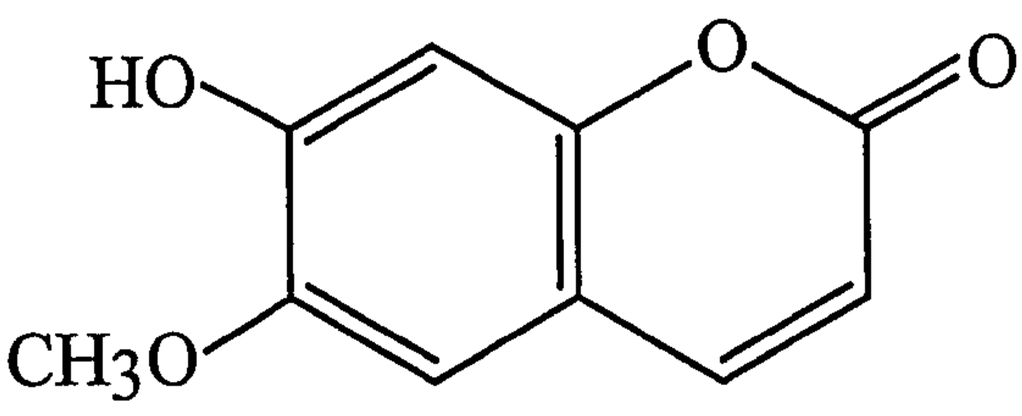
Trigonelline



4-Hydroxyisoleucine



Coumarin



Scopoletin

Fenugreekine

Figure 1.15: Some hypoglycaemic constituents of *Trigonella foenum-graecum*

1.2.5 Models for screening anti-diabetic plants

Ideally, large scale clinical trials should be employed to investigate the efficacy of traditional herbal remedies for the treatment of diabetes. However, this is generally not feasible due to financial, ethical and practical reasons. Instead, various *in vivo* and *in vitro* techniques are used for this purpose, as discussed below.

In vivo techniques for the study of hypoglycaemic activity employ animals with induced hyperglycaemia, either chemically induced i.e. treated with drugs such as streptozotocin or alloxan, or genetically bred (Fischer, 1985; Shafrir, 1990). In fact, many of the studies on anti-diabetic plants involve the use of animal models. *In vivo* bioassays are undoubtedly essential to prove the value of new hypoglycaemic components from plants. However, whole animal tests reveal relatively little about the mode of action of the extracts/compounds studied, and it can be seen from previous sections (Section 1.2.1-1.2.4) that there are many mechanisms by which blood glucose levels may be reduced. Furthermore, the use of *in vivo* bioassay-guided fractionation for isolating active constituents from plants is restricted by the cost of obtaining and maintaining animals, social and ethical issues on extensive use of animals in experimentation, and the requirement of practical training for good technical skills.

Thus a number of *in vitro* models have been developed to elucidate the varied mechanisms of action of hypoglycaemic agents:

- **Inhibition of intestinal glucose uptake**. Studies on glucose uptake into intestinal brush border membrane vesicles (Kessler *et al*., 1978), Caco-2 cells (Neef *et al*., 1996) or everted gut (Perl and Hikino, 1989) to detect compounds that can inhibit intestinal glucose absorption.
- **Stimulation of insulin release**. Isolated pancreatic islets (Jones *et al*., 1993) and insulin-secreting cell lines e.g. HIT-T15 cells (Poitout *et al*., 1996) are used to detect compounds with insulin secretagogue activity.
- **Insulinomimetic or insulin sensitising effects**. Isolated hepatocytes (Quentmeier *et al*., 1993) and hepatoma cell lines e.g. H4IIE (Valera and Bosch, 1994) are employed for detecting compounds with insulinomimetic effects on the liver. Other insulin target tissues such as skeletal muscles and adipocytes are also used

(Welihinda and Karunanayake, 1986). The same tissues may also be used to detect compounds with insulin sensitising effects.

In vitro assays have the advantage of utilising a smaller number of animals (as a source of tissues or cells) and are particularly useful when screening large numbers of plant extracts and chromatographic fractions. However, a wide range of assays are required for the screening of plants for anti-diabetic activity and to elucidate the mode of action of the isolated phytochemicals because each assay is specific for a particular mechanism of action. In addition, caution must be taken when assessing *in vitro* results since compounds in plant extracts showing *in vitro* activity may not always be bioavailable *in vivo*. For instance, some compounds such as proteins/peptides may possess an effect *in vitro* but if administered orally in *in vivo* models, they may have been hydrolysed in the gastrointestinal tract before reaching the site of action. On the other hand, some precursor compounds which require certain metabolic activation processes *in vivo* would have failed to produce effect in *in vitro* models. Furthermore, the *in vitro* behaviour of test tissues may also differ from the *in vivo* situation. Thus any compounds/extracts which show activity *in vitro* must have their activities confirmed using appropriate *in vivo* models.

1.2.6 Anti-diabetic plant research

Many plants have been used as traditional herbal remedies for the treatment of diabetes. Among all these plants, selecting one which has high priority for extensive research study on its anti-diabetic activity is not an easy decision. However, Marles and Farnsworth (1995) have suggested several general criteria for choosing an appropriate candidate:

The plant should -

a) be traditionally used as an anti-diabetic remedy in one or more countries
b) possess experimentally determined hypoglycaemic activity
c) lack detailed information on hypoglycaemic constituents
d) possess experimental evidence for low toxicity
e) have botanical abundance

One plant which appears to fulfil all these criteria is ***Momordica charantia*** **Linn.**, which forms the subject of the current thesis. This species, together with the plant family Cucurbitaceae to which it belongs, will be discussed in more detail in the following sections (Sections 1.3 and 1.4).

1.3 The Cucurbitaceae family

Cucurbitaceae (Table 1.3), the single family within the order Cucurbitales, is a moderately large family of about 118 genera and 825 species (Jeffrey, 1990). All Cucurbitaceae are frost-sensitive and thus the family is confined to the warmer parts of the globe. The family is most abundantly found within the tropics, especially in tropical Africa and the neotropics (Jeffrey, 1980). Members of this family are mostly herbs climbing by tendrils, with abundant sap and very rapid growth.

The family Cucurbitaceae is of great economic importance; many species of this family are widely cultivated for sources of vegetables and fruits (Table 1.4). For example, twelve genera and 18 species of cucurbits are currently under cultivation in China (Walters, 1989; Yang and Walters, 1992). It is interesting to note that the majority of cucurbit fruits sold as vegetables are immature, while the mature fruits of some species are grown for other uses such as for culinary and decorative purposes. One classic example is the species *Lagenaria siceraria*, the immature fruits are cultivated for consumption, while the mature fruits are commonly used as containers and musical instruments (Andres *et al.*, 1996).

1.3.1 The phytochemistry of the Cucurbitaceae

Biochemically, the Cucurbitaceae are characterised by the presence of sterols, cucurbitacins and other triterpenoids in the vegetative parts and fruits, and by the occurrence in the seeds of free amino acids, seed oils and storage proteins (Jeffrey, 1980).

1.3.1(a) Sterols of the Cucurbitaceae

Cucurbitaceae is a family of plants with a reputation for complex steroid chemistry. Studies by previous researchers (Akihisa *et al.*, 1987a) have shown that the sterols found in members of the Cucurbitaceae possessed the following 7 types of skeletons (Fig. 1.16):

1	saturated	**5**	$\Delta^{8(14)}$- unsaturated
2	Δ^{5}- unsaturated	**6**	14α-methyl-9β, 19-cyclo skeleton
3	Δ^{7}- unsaturated	**7**	14α-methyl-$\Delta^{9(11)}$- unsaturated
4	Δ^{8}- unsaturated		

Table 1.3 The taxonomic hierarchy with respect to Cucurbitaceae

Division	Angiospermae		
Class	Dicotyledoneae		
Subclass	Archichlamydeae		
Order	Cucurbitales		
Family	**Cucurbitaceae**		
Subfamilies (2)	Cucurbitoideae (8 tribes; 101 genera)		
	Zanonioideae (17 genera)		
Genera (118)	e.g.	*Bryonia*	(4 species)
		Citrullus	(3 species)
		Cucumis	(25 species)
		Cucurbita	(5 species)
		Ecballium	(1 species)
		Luffa	(6 species)
		Momordica	(45 species)

Table 1.4 The Cucurbitaceae family as a source of vegetables & fruit

Family members	Vegetables/Fruit
Benincasa hispida	winter melon
Benincasa hispida var. *chieh-qua*	hairy melon
Citrullus lanatus	water melon
Citrullus lanatus var. *citroides*	citron
Cucumis anguria	gherkin
Cucumis melo	muskmelon
Cucumis sativus	cucumber
Cucurbita maxima	pumpkin
Cucurbita pepo	vegetable marrow
Ecballium elaterium	squirting cucumber
Lagenaria siceraria	bottle gourd
Luffa acutangula	ridged loofah/silky gourd
Luffa cylindrica	smooth loofah/water gourd
Momordica charantia	bitter melon

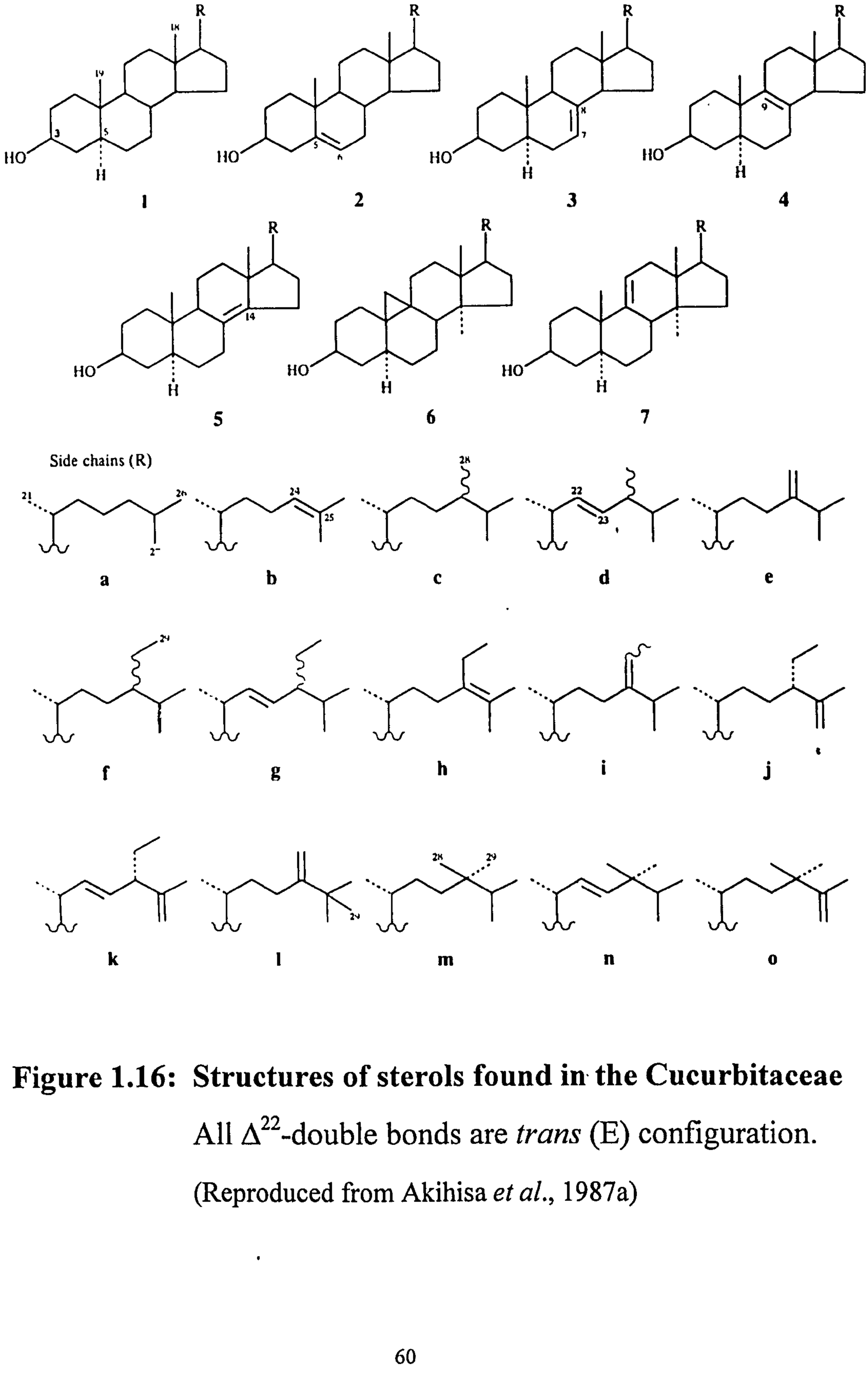


Figure 1.16: Structures of sterols found in the Cucurbitaceae

All Δ^{22}-double bonds are *trans* (E) configuration.

(Reproduced from Akihisa *et al.*, 1987a)

with the side chains (Fig. 1.16) of:

24-unsubstituted (**a**)
Δ^{24}-substituted (**b**)
24-methyl-substituted (**c**)
24-methyl-Δ^{22}-substituted (**d**)
24-methylene-substituted (**e**)
24-ethyl-substituted (**f**)
24-ethyl-Δ^{22}-substituted (**g**)
24-ethyl-$\Delta^{24(25)}$-substituted (**h**)
24-ethylidene-substituted (**i**)
24-ethyl-Δ^{25}-substituted (**j**)
24-ethyl-$\Delta^{22,25}$-substituted (**k**)
24-methylene-25-methyl-substituted (**l**)
24,24-dimethyl-substituted (**m**)
24,24-dimethyl-Δ^{22}-substituted (**n**)
24,24-dimethyl-Δ^{25}-substituted (**o**)

The great majority of higher plants contain predominantly sterols bearing a Δ^5-bond with a 24α-alkyl substituted side chain (24*R* if a saturated or Δ^{25}-unsaturated side chain, 24*S* if the Δ^{22} derivative) represented by sitosterol (24α-**2f**). However, plants of the family Cucurbitaceae contain mainly 24-alkyl-Δ^7-sterols, such as 24-ethylcholest-7-enol (**3f**), 24-ethylcholesta-7,22-dienol (**3g**), 24-ethylcholesta-7,25-dienol (**3j**) and 24-ethylcholesta-7,22,25-trienol (**3k**), which constitute the characteristic feature of the plants of this family (Akihisa *et al.*, 1987a). Akihisa *et* al. (1987a) also reported that among the 12 genera of the Cucurbitaceae investigated, 44 sterols were identified. The Δ^7-sterols (**3c-3k**) were found to be the major sterols which were accompanied by small amounts of the sterols with saturated (**1c, 1f, 1g**) and Δ^5- (**2a-2g, 2i-2k**) and Δ^8- (**4g, 4j, 4k**) skeletons. The 24-ethyl-$\Delta^{7,22}$- (**3g**), $\Delta^{7,25(27)}$- (**3j**) and $\Delta^{7,22,25(27)}$- (**3k**) sterols constituted the predominant sterols for the seed materials, whereas the 24-ethyl-Δ^7- (**3f**) and $\Delta^{7,22}$- (**3g**) sterols were the major ones for the mature plant tissues which consisted of leaves and stems, pericarp of the fruit and roots (Akihisa *et al.*, 1986a,b). In addition, several new or uncommon sterols were found as minor constituents, for example, 24-methylene-25-methylcholest-7-enol (**3l**) in *Sicyos angulatus* L. (Akihisa *et al.*, 1987b); 24,24-dimethylcholest-7-enol (**3m**), 24,24-dimethylcholesta-7,22-dienol (**3n**) and 24,24-dimethylcholesta-7,25-dienol (**3o**) in *Gynostemma pentaphyllum* Makino (Akihisa *et al.*, 1986c); 24α-ethylcholesta-8(14),22-dienol (24α-**5g**) (Akihisa *et al.*, 1986a) and 24-methylenepollinastanol (**6e**) and 14α-methyl-24α-ethylcholest-9(11)-enol (24α-

7f) (Akihisa *et al.*, 1986d) in *Cucumis sativus* L.

An extensive investigation on the configurations at C-24 of the 24-alkylsterols showed that the 24-methyl- and 24-ethylsterols lacking a Δ^{25}- bond (i.e. 24-methyl-, 24-methyl-Δ^{22}-, 24-ethyl- and 24-ethyl-Δ^{22}-sterols) occurred as the C-24 epimeric mixtures, or as a single diastereoisomer, either 24α- and 24β-epimer. On the other hand, the 24-ethylsterols bearing a Δ^{25}-bond (i.e. 24-ethyl-Δ^{25}- and 24-ethyl-$\Delta^{22,25}$-sterols) were composed of 24β-epimers only (Akihisa *et al.*, 1987a).

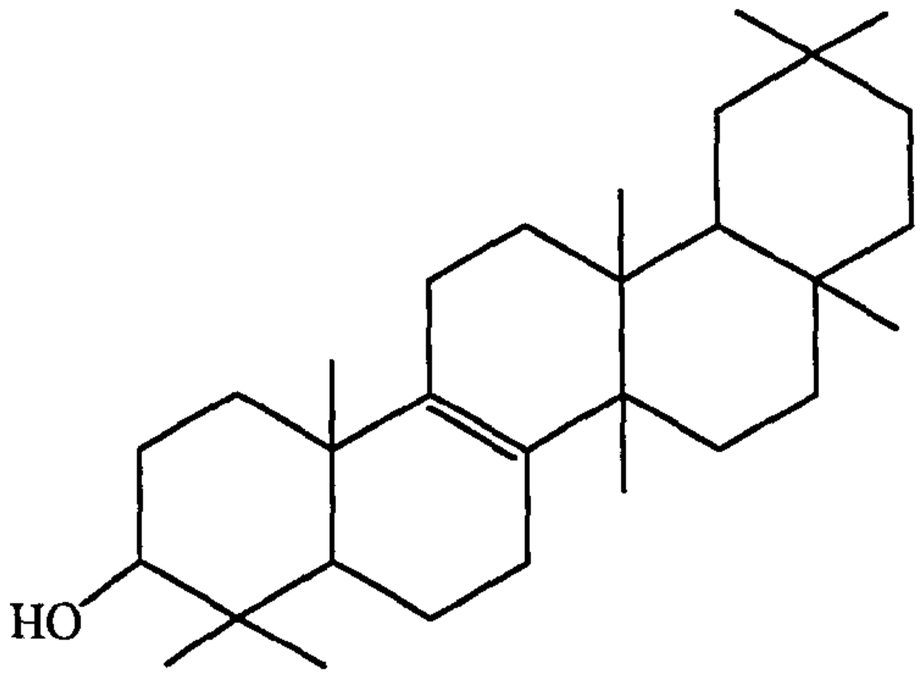
1.3.1(b) Triterpene alcohols of Cucurbitaceae

Among the 13 genera of the family Cucurbitaceae examined by Akihisa *et al.* (1988), 14 tetracyclic and pentacyclic triterpene alcohols were identified. Isomultiflorenol (Fig. 1.17) was found to be the major component in the seeds, which suggested that it may be a taxonomical marker of the seeds of Cucurbitaceae since only a few higher plants are known to contain this triterpene. The mature plant materials (aerial parts, pericarp of the fruits, and roots) of the Cucurbitaceae were shown to contain either one or more of the common triterpene alcohols such as α- and β-amyrins, cycloartenol and 24-methylenecycloartanol as the major triterpene constituents (Fig. 1.17).

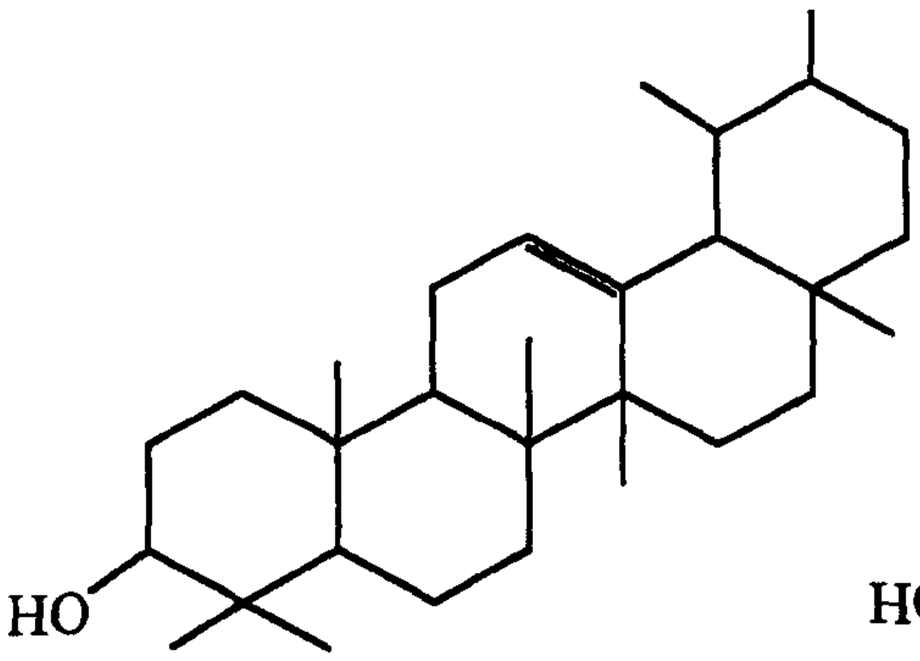
1.3.1(c) Cucurbitacins

Cucurbitacins are predominantly found in the Cucurbitaceae family but are also present in several other plant families such as Begoniaceae, Cruciferae, Desfontainiaceae, Elaeocarpaceae and Scrophulariaceae (Miró, 1995).

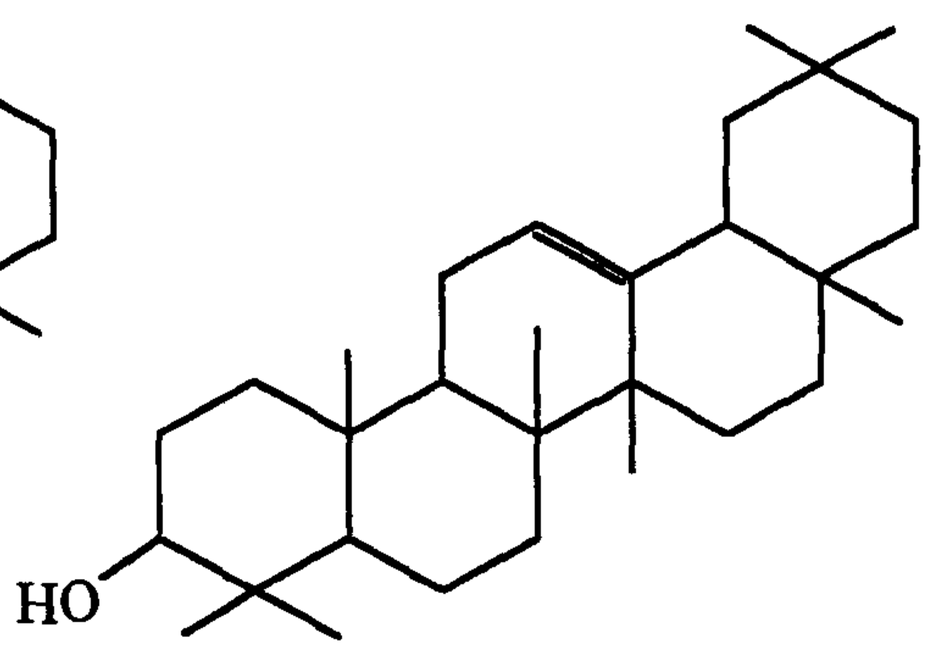
Cucurbitacins are a group of bitter tasting, highly oxygenated, mainly tetracyclic, triterpenes (possessing 30 carbons), derived from the cucurbitane skeleton [19-(10→9β)-abeo-10α-lanost-5-en] as shown in Fig. 1.18. The C_4 carries a gem-dimethyl group and the C_{24} an isopropyl group, while other methyls are present on C_9, C_{13}, C_{14} and C_{20}. All of the cucurbitacins have an unsaturated C_5 position. Cucurbitacins cannot be considered as steroidal since the methyl group has moved from C_{10} to C_9.



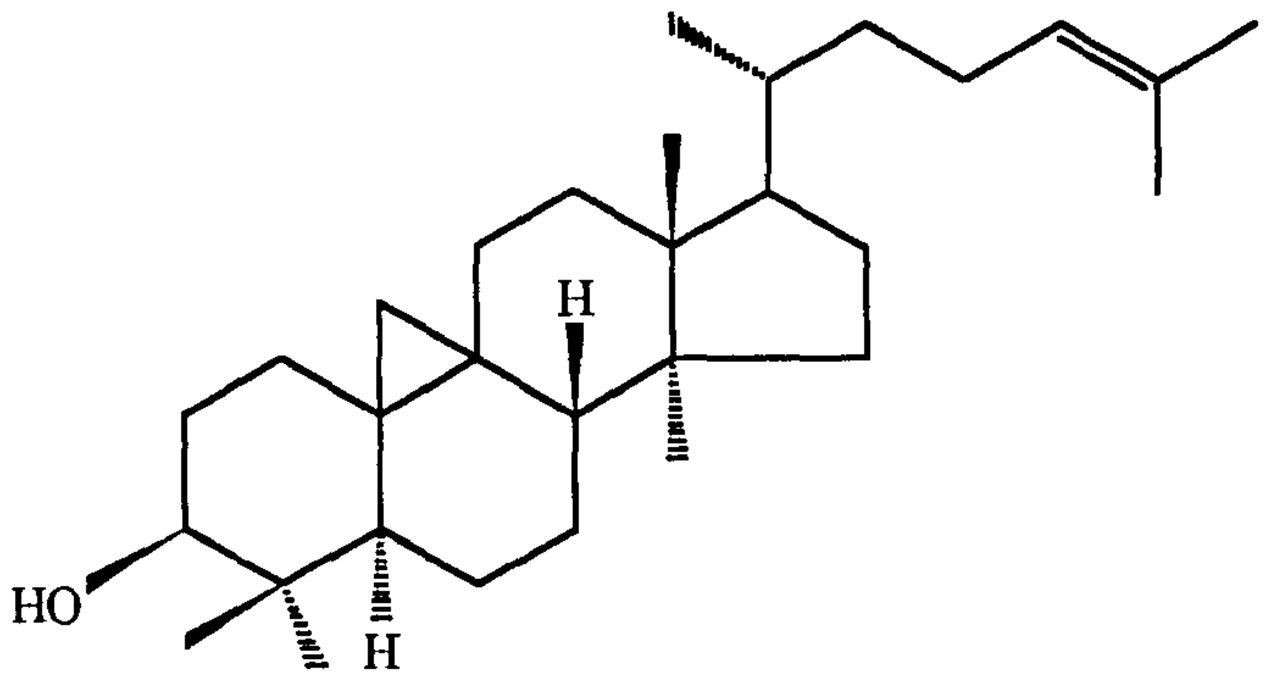
Isomultiflorenol



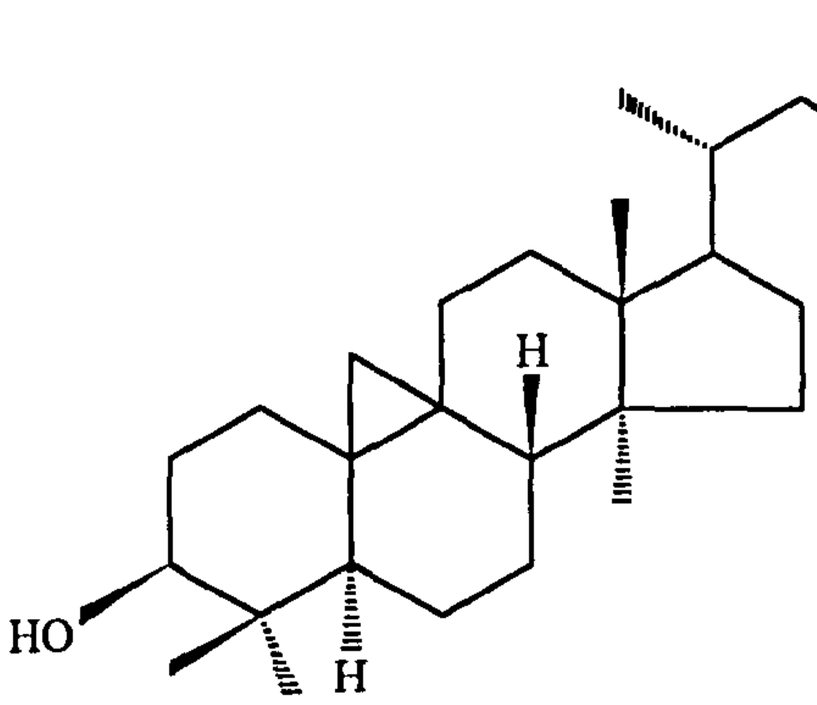
α-Amyrin



β-Amyrin



Cycloartenol



24-Methylenecycloartanol

Figure 1.17: Structures of some triterpene alcohols found in Cucurbitaceae

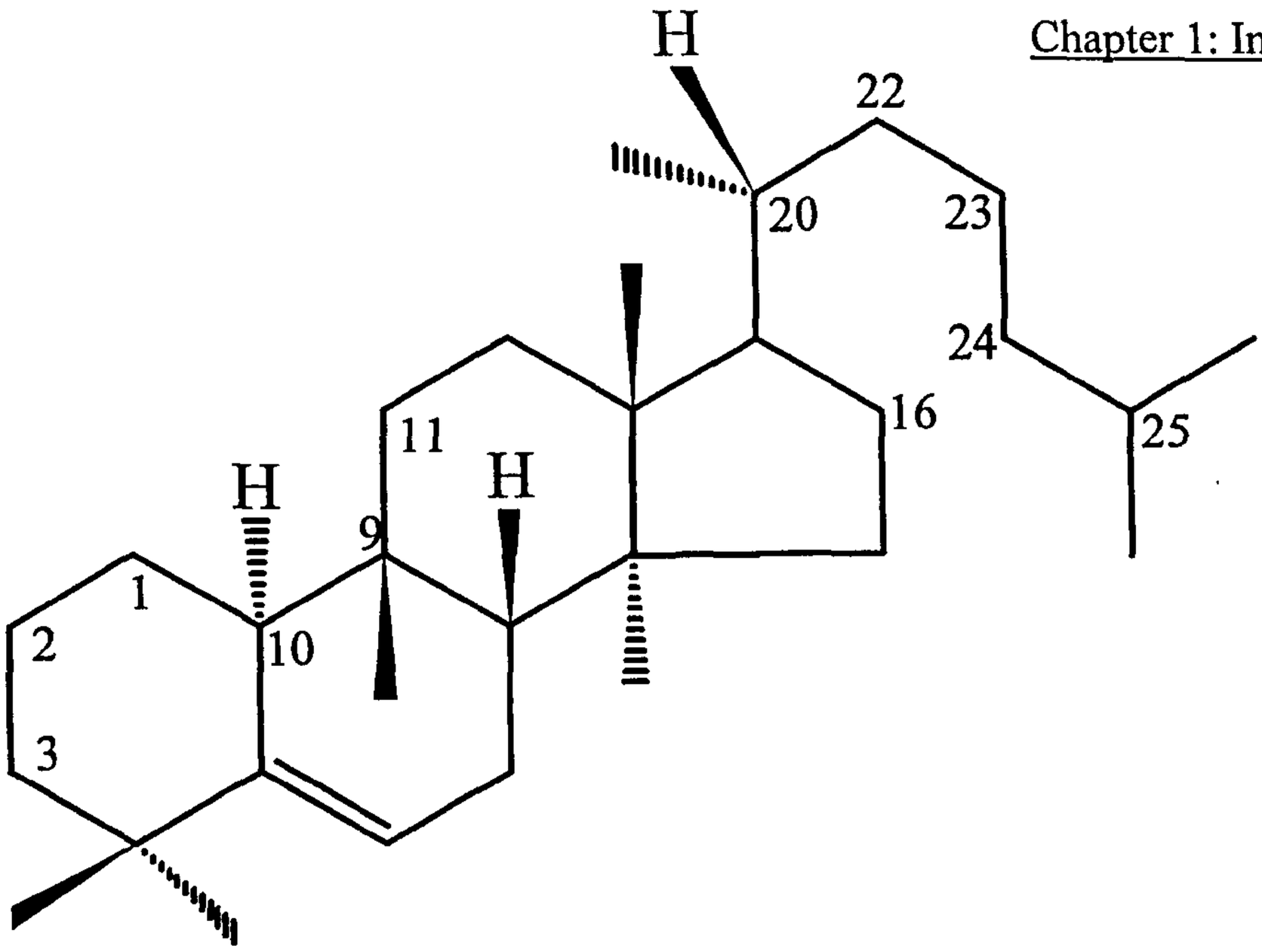


Figure 1.18: General structure of cucurbitacins

The most common substituents are as follows:

C_1: Sometimes unsaturated (e.g. cucurbitacins E, I, J, K and L)

C_2: Usually hydroxylated except in iso forms and in cucurbitacin C which has no substituents.

C_3: Hydroxylic or ketonic

C_9: Usually methylated except in cucurbitacins A and C where it is hydroxymethylated

C_{11}: Ketonic except in 11-deoxo-cucurbitacin I

C_{16}: Hydroxylic (α)

C_{20}: Hydroxylic (β)

C_{22}: Ketonic except in 22-deoxo and 22-deoxo-isocucurbitacin D

C_{23}: Sometimes unsaturated (e.g. cucurbitacins A, B, C, D, E, F, I, O and Q)

C_{24}: Not usually substituted except for a hydroxyl group on cucurbitacins G, H, J and K.

C_{25}: Can be hydroxylated or acetoxylated

Cucurbitacins are widely distributed in the plant, being found in highest concentration in the seeds and roots. Their intense bitterness means that they are effectively defensive against many herbivores. However, they are not always bitter tasting since sweet cucurbitane glycosides, e.g. Carnosiflosides V and VI, have been isolated from the rhizomes of *Hemsleya carnosiflora* (Kasai *et al.*, 1987).

The cucurbitacins were all named after successive letters of the alphabet from A to R, and at a later stage, another two cucurbitacins S and T, isolated from *Bryonia dioica* (Hylands and Salama, 1976; Hylands and Mansour, 1982), which present a new heptagonal ring between carbons C_{16} and C_{24} were added. Cucurbitacins are usually present in the plant as β-glucosides, which are hydrolysed by the enzyme elaterase to give the free genins.

Cucurbitacins represent an important phytochemical group due to their various pharmacological effects and their possible clinical applications (Miró, 1995), as listed below.

Cytotoxic and antitumour action

Cucurbitacins B, D, E, I, J, K, L and tetrahydro-I isolated from *Bryonia alba* roots all revealed cytotoxic activity in cell cultures. The cytotoxic action mechanism seems to involve a blockage in the incorporation of thymidine by the nucleic acids.

Hepatoprotective and hepatocurative activities

Cucumis melo, containing cucurbitacins B and E, is a remedy in Chinese traditional medicine for hepatitis. Cucurbitacin B has been shown to be effective in chronic hepatitis by normalising hepatic protein levels, stimulating cellular immunity functions and increasing the plasma cAMP/cGMP ratio in experimental animals. Cucurbitacin E also increases the cAMP/cGMP ratio.

Antimicrobial activity

The cucurbitacin containing species, *Momordica charantia*, possesses antibacterial activity (Fatope *et al.*, 1990). In particular, cucurbitacin C at a concentration of 10mg/mL inhibits growth of the fungus, *Phytophtora cactorium*.

Anti-inflammatory activity

Cucurbitacin B, isolated from the juice of *Ecballium elaterium*, was shown to exhibit

significant anti-inflammatory action. It may work by inhibiting eicosanoid synthesis which modifies the production of leukotriene B_4.

Other pharmacological effects of cucurbitacins include their strong purgative properties and antihelminthic activity. They can also play other biological roles such as plant growth regulators and insect feedants (Metcalf and Lampman, 1989) or antifeedants.

1.3.1(d) Seeds of the Cucurbitaceae

Apart from the presence of sterols and cucurbitacins, eight free amino acids have been identified in the seeds of Cucurbitaceae, as well as a large number of other ninhydrin-positive compounds, the major one is citrulline which has been found in all genera investigated (Dunhill and Fowden, 1965). The role of these free amino acids and ninhydrin-positive compounds is not known, however they may possibly function as chemical defence mechanisms.

The nutritive value of the seeds stems from their high oil and protein contents. The seed oils of Cucurbitaceae fall into 2 groups: one with palmitic-oleic-linoleic acid composition, the other with conjugated triene acids - punicic acid and α-eleostearic acid (Jeffrey, 1980). Badifu (1991) reported that among the species of Cucurbitaceae examined, the main fatty acids found in the seed oils were palmitic, stearic, oleic and linoleic. The seed proteins of Cucurbitaceae are high in nutritive value, though they tend to be low in lysine and sulphur-containing amino acids. They are generally rich in methionine (Jeffrey, 1980).

1.3.2 Cucurbitaceae and diabetes mellitus

A recent article by Marles and Farnsworth (1995) revealed that 30 species (belonging to 11 genera) of plants within the Cucurbitaceae family have either been used ethnomedically to treat diabetes mellitus or have been tested for therapeutic activity against diabetes mellitus (Table 1.5). One such example is *Momordica charantia* L., which is the subject of our present study. In the following section, an extensive and up to date literature review on the anti-diabetic properties and known phytochemical constituents of *Momordica charantia* L. will be presented.

Table 1.5 A list of traditionally used anti-diabetic plants which belong to the Cucurbitaceae family (Marles and Farnsworth, 1995)

Benincasa hispida	*Bryonia alba*
Bryonia cretica	*Bryonia dioica*
Bryonia epigaea	*Citrullus colocynthis*
Citrullus lanatus	*Coccinia cordifolia*
Coccinia grandis	*Coccinia indica*
Cucumis melo	*Cucumis sativus*
Cucurbita maxima	*Cucurbita moschata*
Cucurbita pepo	*Lagenaria siceraria*
Lagenaria vulgaris	*Luffa acutangula*
Luffa echinata	*Melothria heterophylla*
Momordica balsamina	*Momordica charantia*
Momordica cochinchinensis	*Momordica foetida*
Trichosanthes anguina	*Trichosanthes bracteata*
Trichosanthes cucumeroides	*Trichosanthes dioica*
Trichosanthes kirilowii	*Trichosanthes multiloba*

1.4 Anti-diabetic properties and phytochemistry of *Momordica charantia* Linn. (Cucurbitaceae)

According to Engler's scheme, the systematic position of *Momordica charantia* in the botanical taxonomy is illustrated below:

Division	Angiospermae
Class	Dicotyledoneae
Subclass	Archichlamydeae
Order	Cucurbitales
Family	Cucurbitaceae
Subfamily	Cucurbitoideae
Tribe	Joliffieae
Genus	*Momordica*
Species	*Momordica charantia* Linnaeus

The unripe fruit of *Momordica charantia* Linn. has been used in Ayurvedic (Indian) and Chinese medicine as well as in the West Indies for the treatment of diabetes. In fact, the unripe fruit and seeds of *Momordica charantia* L. have been the subject of over a hundred scientific articles describing their pharmacological or phytochemical properties (Raman and Lau, 1996). Thus this section aims to review the scientific investigations conducted so far on *Momordica charantia* with respect to its anti-diabetic use in traditional remedies and to explore the possibility of future work in this area which may lead to the development of new anti-diabetic agents.

1.4.1 Habitat and traditional uses

The native country of *Momordica charantia* is uncertain, but the plant is cultivated throughout the tropics, particularly in India, China, East Africa and Central and South America (Walters and Decker-Walters, 1988). It is occasionally grown as an ornamental creeper, but more commonly cultivated for use of the unripe fruit as a vegetable. This has led to the existence of a wide variety of cultivars. The fruit has a number of different local names, being well-known as karela (India), also named bitter gourd, bitter-melon, balsam-pear, cundeamor (South America), ku-gua (China), carilla or goo-fah (Jamaica); the reported spelling of the local names is often variable.

The wild variety (*M. charantia* var. *abbreviata*) grows as a weed in the West Indies, where the plant is known as cerasee (Jamaica) or sorossie (Dominican Republic). This variety has smaller fruit than the Indian one. The term karela is used throughout this section to denote all varieties of the fruit since in the majority of studies the type used has not been specified.

In addition to its major use as an anti-diabetic agent, karela has been used in India and Sri Lanka as a tonic, emetic and laxative (Nadkarni 1982). Both the cultivated and wild forms are used for this purpose (Bailey *et al.*, 1986). In South/Central America, cerasee fruit or tea is used for diabetes, colds and fevers, stomach aches, constipation in children and the induction of abortion (West *et al.*, 1971; Arvigo and Balick, 1993). Traditional Chinese uses for the fruit, seeds, vines and leaves include gastroenteritis, diabetes, tumours and some viral infections (Zhang, 1992a).

When used as an anti-diabetic remedy, karela juice prepared by crushing and straining the unripe fruit (ca. 50ml) is taken once or twice a day. Fried karela may also be consumed. Cerasee on the other hand, is taken as a decoction or "tea" (hot water extract) of the aerial parts of the plant, free of large fruit (Bailey *et al.*, 1986).

1.4.2 Studies in human subjects

Karela fruit. To date, no large scale clinical trial has been reported on the anti-diabetic effects of karela, but a number of studies using small groups of diabetic patients have been conducted. Both non-insulin-dependent diabetes mellitus (NIDDM, Type II, maturity onset) and insulin-dependent (IDDM, Type I, juvenile onset) patients have participated.

Kirti *et al.* (1982) have described some early studies (1950-1974) carried out in India and the Caribbean, in which karela's anti-diabetic activity was observed. More recent interest was aroused when Aslam and Stockley (1979) reported a case of a possible interaction, in the form of decreased glycosuria, between the anti-diabetic drug chlorpropamide and a curry containing karela, taken by an Asian NIDDM patient. Following this, Leatherdale *et al.* (1981) carried out a study in 9 Asian NIDDM outpatients living in the United Kingdom, who were being treated with diet alone or diet combined with oral hypoglycaemic agents. Acute administration of karela juice

with a glucose load resulted in a significant improvement in glucose tolerance without increasing the insulin levels in the blood. Daily consumption of fried karela for 8 to 11 weeks had a similar, though not statistically significant, effect. Nevertheless, there was a significant reduction in glycosylated haemoglobin, indicating an improved control of blood glucose levels over this period.

Further evidence for a beneficial chronic effect is that an improvement in both glucose tolerance, and fasting blood glucose levels was observed in 8 NIDDM patients following 7 weeks of daily consumption of powdered karela fruit (Akhtar, 1982). Srivastava *et al.* (1993) reported that 3-7 weeks treatment of diabetics with powdered fruit, led to a mean fall of 25% (range 11-48%) in post-prandial blood glucose levels. There was a marked fall in both blood and urine sugar over 7 weeks in a group treated with an aqueous extract of the fruit. Glycosylated haemoglobin showed a significant reduction by the end of the trial.

By contrast, Kirti *et al.* (1982) reported that whilst karela (acute or chronic administration) resulted in a reduction in glycosuria, there was no effect on blood glucose. However, in their experiments, blood glucose levels were measured two hours after the administration of karela extract and by this time any effects of the fruit may have diminished. The earlier work of Leatherdale *et al.* (1981) suggested that improved glucose tolerance is most marked within the first 90 minutes of karela administration. Inter-patient variation may also explain a poor response to karela; Welihinda *et al.* (1986) reported that karela juice significantly improved glucose tolerance in only 13 of the 18 patients tested.

P-insulin. In 1974, Khanna *et* al. isolated a polypeptide (polypeptide p, p-insulin or v-insulin; p = plant, v = vegetable) from karela. A significant hypoglycaemic effect was observed in 6 IDDM, 1 NIDDM and 2 asymptomatic diabetics administered p-insulin subcutaneously (Baldwa *et al.*, 1977). In a later study by Khanna *et al.* (1981), subcutaneous p-insulin led to a significant fall in blood glucose in 11 IDDM patients, whereas a similar effect in 8 NIDDM patients did not reach statistical significance. P-insulin from karela therefore appears to have insulin-like effects. One IDDM patient was reported to have been maintained on p-insulin for 5 months with no complaints of side effects.

Karela seeds. As well as the fruit, the potential anti-diabetic effects of the seeds have been examined. Oral administration of powdered karela seeds produced a significant reduction in post-prandial blood sugar values in 14 NIDDM and 6 IDDM patients (Grover and Gupta, 1990).

1.4.3 In vivo studies in laboratory animals

There have been numerous studies on karela in laboratory animals *in vivo*. Studies using laboratory animals have included normal animals of various species, and those in which diabetes mellitus has been induced by administration of alloxan or streptozotocin (STZ). These two drugs are known to selectively damage insulin-secreting beta cells of the pancreas, resulting in partial or virtual loss of insulin production, and their effects are irreversible (Fischer, 1985). On the other hand, the antihistaminic drug cyproheptadine which produces a reversible loss of pancreatic insulin when given in repeated doses (Fischer, 1985), is also used to induce diabetes in laboratory animals. No reports of the action of karela on genetically diabetic animals have been found to date.

Karela juice or extracts. The three main animal species in which the effects of karela juice or karela solvent extracts have been investigated are the rabbit, rat and mouse.

Rabbit model. One of the earliest reports of karela's activity was by Sharma *et al.* (1960) who reported that the juice caused an improvement in glucose tolerance in alloxan diabetic but not normal animals. Somewhat in contrast to this, Akhtar *et al.* (1981) found that dried karela fruit caused a significant dose dependent decrease in blood glucose and that a higher minimum dose was required for alloxan-treated rabbits than normal ones. The effectiveness in alloxan diabetic animals suggests that extra-pancreatic effects are involved; and since a higher minimum dose of karela was required to elicit a response in these animals, pancreatic stimulation may play some part in the effects of the fruit. However, Kulkarni and Gaitonde (1962) saw no reduction in fasting glucose levels on either acute or chronic administration of dried karela juice to normal rabbits. These conflicting results may be due to variations in blood sampling times and dosages of both karela and alloxan.

A number of solvent extracts of karela have also been tested. Intravenous administration of a chloroform soluble extract of the juice resulted in a marked hypoglycaemic effect in alloxan-treated but not normal rabbits (Tiangda *et al.*, 1987). This may be an indication of greater pancreatic β-cell sensitivity to karela in alloxan-treated animals. Glucose tolerance in alloxan recovered rabbits (rabbits that had recovered normal fasting blood glucose levels but still showed inadequate glucose tolerance) was improved by oral administration of a benzene extract of karela, but not an ethanolic one (Venkanna Babu *et al.*, 1988). Three non-sapogenic hypoglycaemic and one hyperglycaemic principles were reported to have been isolated from karela, but their identities were not given.

Rat model. Rat models have been widely used to study the effects of karela juice and its extracts. Improved glucose tolerance on acute administration of the juice has been demonstrated in normal rats (Karunanayake *et al.*, 1984; Chandrasekar *et al.*, 1989) and in rats with anterior pituitary extract-induced hyperglycaemia (Gupta, 1963). Chronic administration over 30 days lowered the mean glucose tolerance in a group of STZ-treated rats, but this did not reach statistical significance (Karunanayake *et al.*, 1990).

Higashino *et al.* (1992) found that a polar solvent extract of karela improved tolerance of both orally and intraperitoneally administered glucose, suggesting that a mechanism involving an extra-intestinal action, though impaired glucose absorption may also be involved. Ali *et al.* (1993a) demonstrated that improved glucose tolerance only occurred in NIDDM-model STZ-treated rats and not those in which IDDM had been induced with a higher dose of STZ. This suggests an insulin secretagogue activity by karela. However, Leatherdale *et al.* (1981) found no significant increase in insulin levels in response to karela treatment in normal rats.

In a recent study, Sarkar *et al.* (1996) reported that an alcoholic extract of karela significantly depressed plasma glucose levels by 10-15% at 1 hour in normal glucose primed rat model, though no increase in insulin secretion was observed. While using STZ-induced diabetic rat model, karela extract improved oral glucose tolerance causing significant reduction in plasma glucose of 26% at 3.5 hour as compared to metformin (reference hypoglycaemic drug) which caused 40-50% reduction at 1, 2

and 3.5 hours. Karela extract was also shown to cause a 4-5 fold increase in the rate of glycogen synthesis from U-^{14}C-glucose in the liver of normal rats. These data suggested that the mechanism of action of karela could be partly attributed to increased glucose utilisation in the liver rather than an insulin secretion effect.

As well as improved glucose tolerance, a hypoglycaemic effect on acute administration of karela juice in fasted rats has been demonstrated in both normal (Leatherdale *et al.*, 1981; Karunanayake *et al.*, 1984; Chandrasekar *et al.*, 1989) and STZ-treated animals (Higashino *et al.*, 1992). However, Ali *et al.* (1993a) found that very high doses of STZ can abolish the effect of karela on basal glycaemia. In alloxan-induced diabetic rats, chronic administration of karela for 20 days was found to lower blood glucose significantly in a dose dependent manner (Srivastava *et al.*, 1987, 1988, 1993). However, Platel *et al.* (1993) found that 8 week administration of freeze-dried fruit to normal animals did not affect blood glucose levels, possibly due to the operation of normal homeostatic mechanisms. In a latter study by Platel and Srinivasan (1995), 6 weeks administration of a semi-synthetic diet containing freeze-dried karela powder to STZ-induced diabetic rats did not affect blood glucose levels and other diabetes related metabolites. In addition, karela juice administered prior to alloxan did not protect the animals from the induction of hyperglycaemia (Sharma *et al.*, 1960).

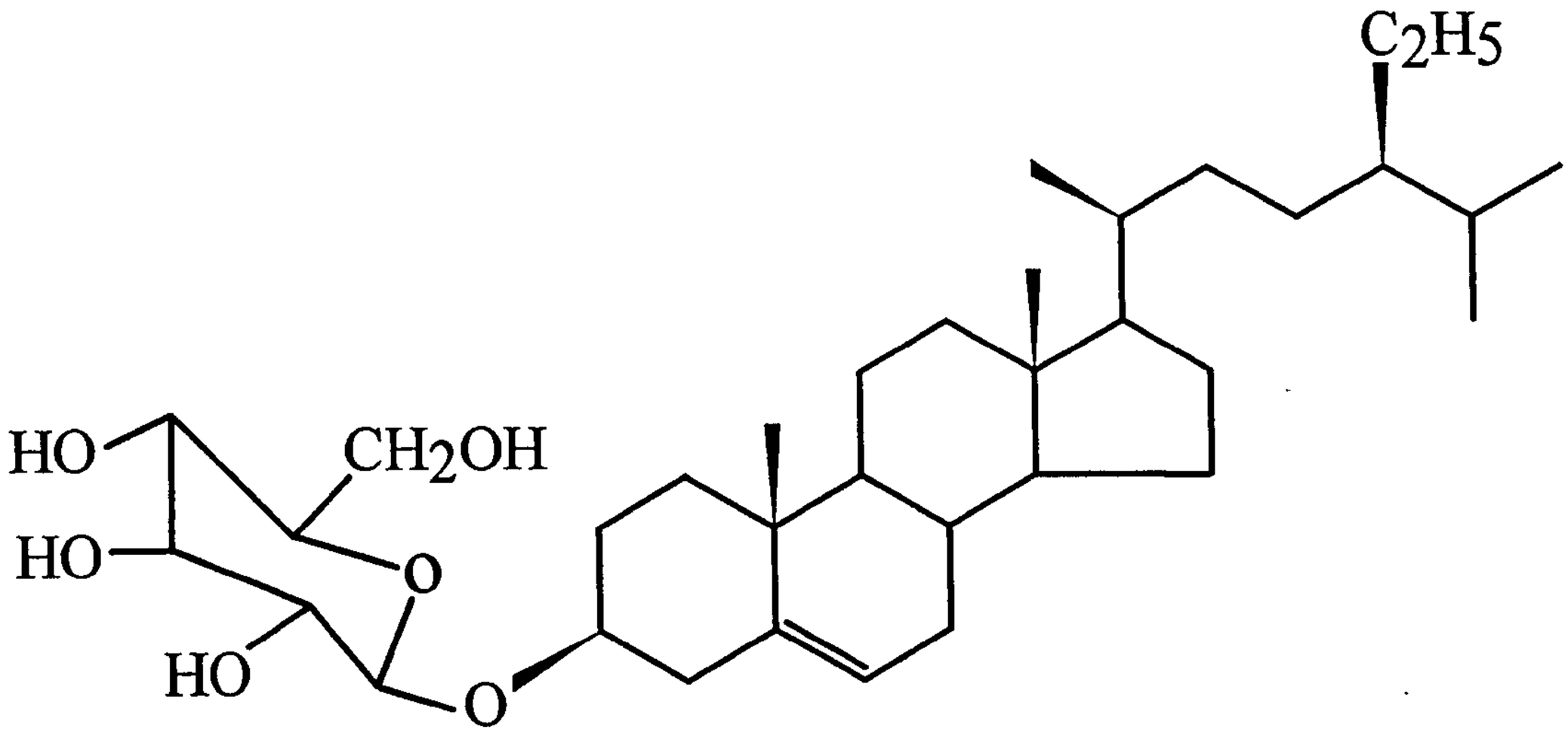
Other potentially beneficial effects of karela administration include lowering of serum cholesterol in normal rats (Platel *et al.*, 1993), in NIDDM model rats (Murshed *et al.*, 1996) and in alloxan-induced diabetic rats (Singh *et al.*, 1989), delaying cataractogenesis in STZ-diabetic animals (Srivastava *et al.*, 1987, 1988, 1993) and normalising the effects on the hepatic cytochrome P450 drug-metabolising enzymes in STZ-induced diabetic rats (Raza *et al.*, 1996).

Mouse model. In normal mice, treatment with karela extracts resulted in improved glucose tolerance using either orally or intraperitoneally administered glucose. There was no significant difference observed between insulin levels in treated and control animals (Day *et al.*, 1990). A hypoglycaemic effect of the juice in STZ-treated animals was also demonstrated. The results of fractionation studies implied the presence of more than one active component, possibly alkaloidal in nature (structure

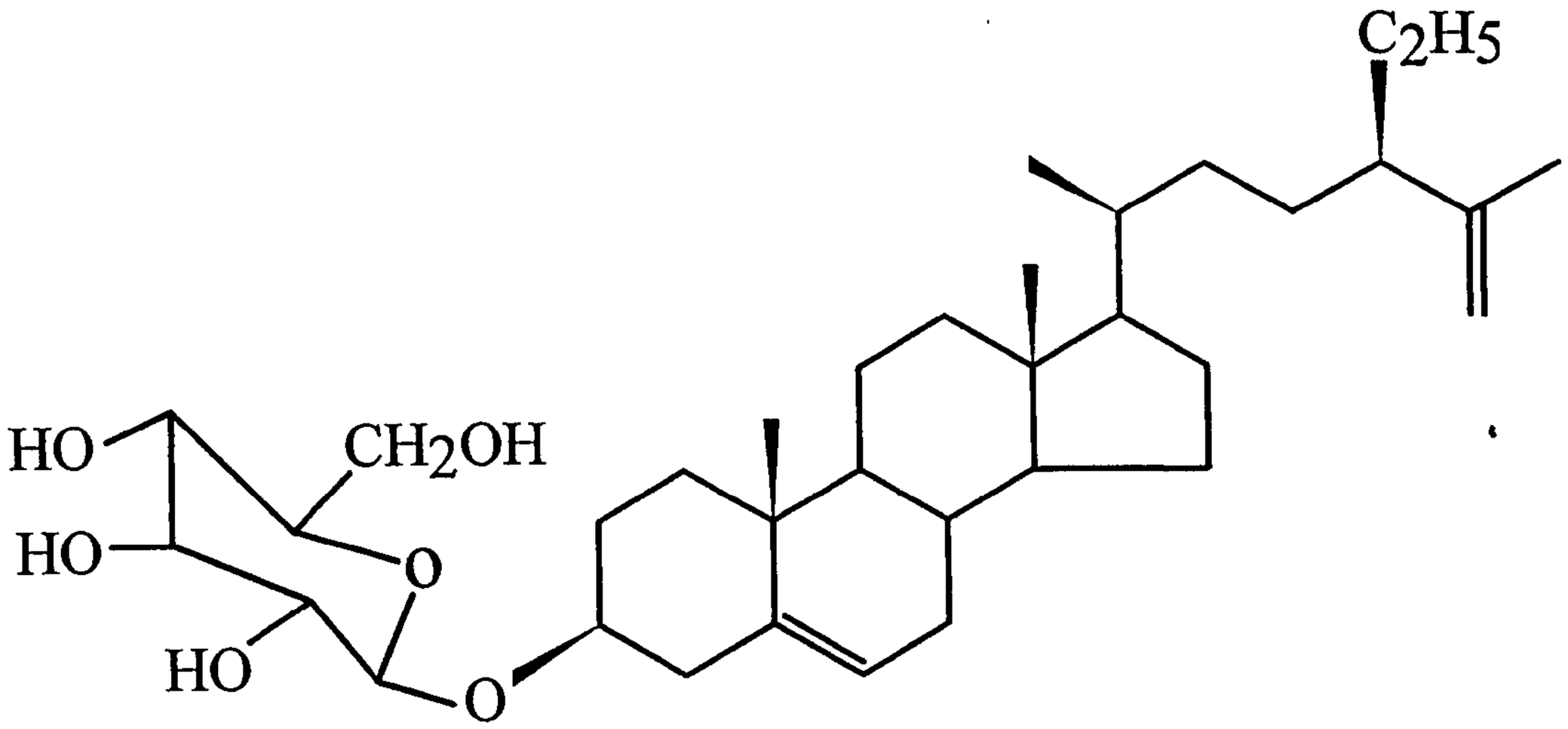
not given). However, Cakici *et al.* (1994) reported that an aqueous extract of karela has a hypoglycaemic activity without improving glucose tolerance in cyproheptadine-induced diabetic mice, suggesting that karela acts independently of intestinal glucose absorption and that an extrapancreatic effect may be involved.

P-insulin. Subcutaneous administration of a polypeptide, p-insulin (isolated from karela) to fasted gerbils and langurs caused a significant fall in blood glucose (Khanna *et al.*, 1981). In another study, Jiangxin *et al.* (1991) reported that both subcutaneous and oral administration of purified p-insulin extracted from karela had significant hypoglycaemic effects in diabetic rats. This is the first and only report found on oral hypoglycaemic activity of p-insulin. However, on closer examination of the results obtained, it was found that the plasma glucose levels before treatment were unexpectedly different between the control and treated (p-insulin) groups, with significantly higher values in the treated group. Thus the results appear to be unreliable and the reported oral hypoglycaemic activity of p-insulin requires further investigation.

Charantin. Charantin (Fig. 1.19), a mixture of β-sitosterol and stigmastadienol glucosides was isolated from karela by Lotlikar and Rajarama Rao (1960/61) in approximately 0.01% yield. A decrease in blood glucose concentration was found when charantin was administered to fasted normal rabbits orally or intravenously. However, the data were obtained using only one or two animals at each dosage level and no controls were carried out. The wide variations in fasting blood glucose values suggest that the animals may not have reached a true fasting state. Furthermore, the dose of charantin administered is equivalent to much higher amounts of karela than those required for a hypoglycaemic response in rabbits, suggesting that charantin is unlikely to account for all the hypoglycaemic activity of the fruit. In a more elaborate study (Lotlikar and Rajarama Rao, 1966), charantin (again at high dose) administered to normal rabbits intravenously or orally produced a gradual but significant fall in blood sugar. In alloxan diabetic rabbits, the effects were more erratic. Pancreatectomy of cats was reported to reduce but not abolish the hypoglycaemic effect of charantin (Lotlikar and Rajarama Rao, 1966), indicating a dual (pancreatic and extra-pancreatic) mechanism of action.



Sitosterol-β-D-glucoside



5,25-Stigmastadienol β-D-glucoside

Figure 1.19: Sterol glucoside components of charantin isolated from *Momordica charantia* fruit

Karela seed. As in human subjects (Grover and Gupta, 1990), karela seed was found to lower blood glucose levels in STZ-induced diabetic rabbits (Kedar and Chakrabarti, 1982). The seed also reversed the low muscle and liver glycogen and elevated serum cholesterol, fatty acids and triglycerides induced by STZ. In a more recent study (Dubey et al., 1987), polar solvent (methanol, 50% aqueous ethanol, normal saline) extracts of karela seed caused a significant hypoglycaemic effect in fasted normal rats. The methanol and saline extracts were also able to reduce adrenaline-induced hyperglycaemia. In both cases, the methanol extract was the most potent. However, Ali *et al.* (1993a) reported that a methanolic extract of the seed did not reduce blood glucose levels in fasting or post-prandial states in normal and STZ-treated IDDM rats.

Vicine. Vicine (Fig. 1.20) has been isolated from the seeds of karela in 0.6% yield (Handa *et al.*, 1990). Intraperitoneal administration of vicine (dose of 100mg/kg body weight) caused a hypoglycaemic response in normal fasting albino rats. The dose used was equivalent to about 16g powder per kg body weight - about five times the dose administered orally by Kedar and Chakrabarti (1982) to obtain a response. This suggests that the activity of the seed powder may not be solely ascribable to vicine.

Karela vines and aerial parts. Cerasee tea (prepared from the vines) was found to lower basal glucose concentrations and to improve glucose tolerance in normal mice (Bailey *et al.*, 1985), with no significant change in the plasma insulin level. A hypoglycaemic response was also observed in STZ-treated mice. When cerasee tea was substituted for drinking water for 12 days, glucose tolerance measured on day 13 was improved. More recently, Ali *et al.* (1993a) tested methanolic and saponin-free methanolic extracts of the whole plant of *Momordica charantia* in normal rats. No effects were seen on fasting blood glucose levels.

1.4.4 Effects on tissues and enzymes; possible mode of action

Attempts have been made to obtain further information on the mode of action of karela fruit and seeds through experiments using enzymes, tissues or cells *in vitro* or examining organs isolated from karela-treated animals. Karela fruit and seed preparations have a number of biological effects *in vitro* (Table 1.6), which may give

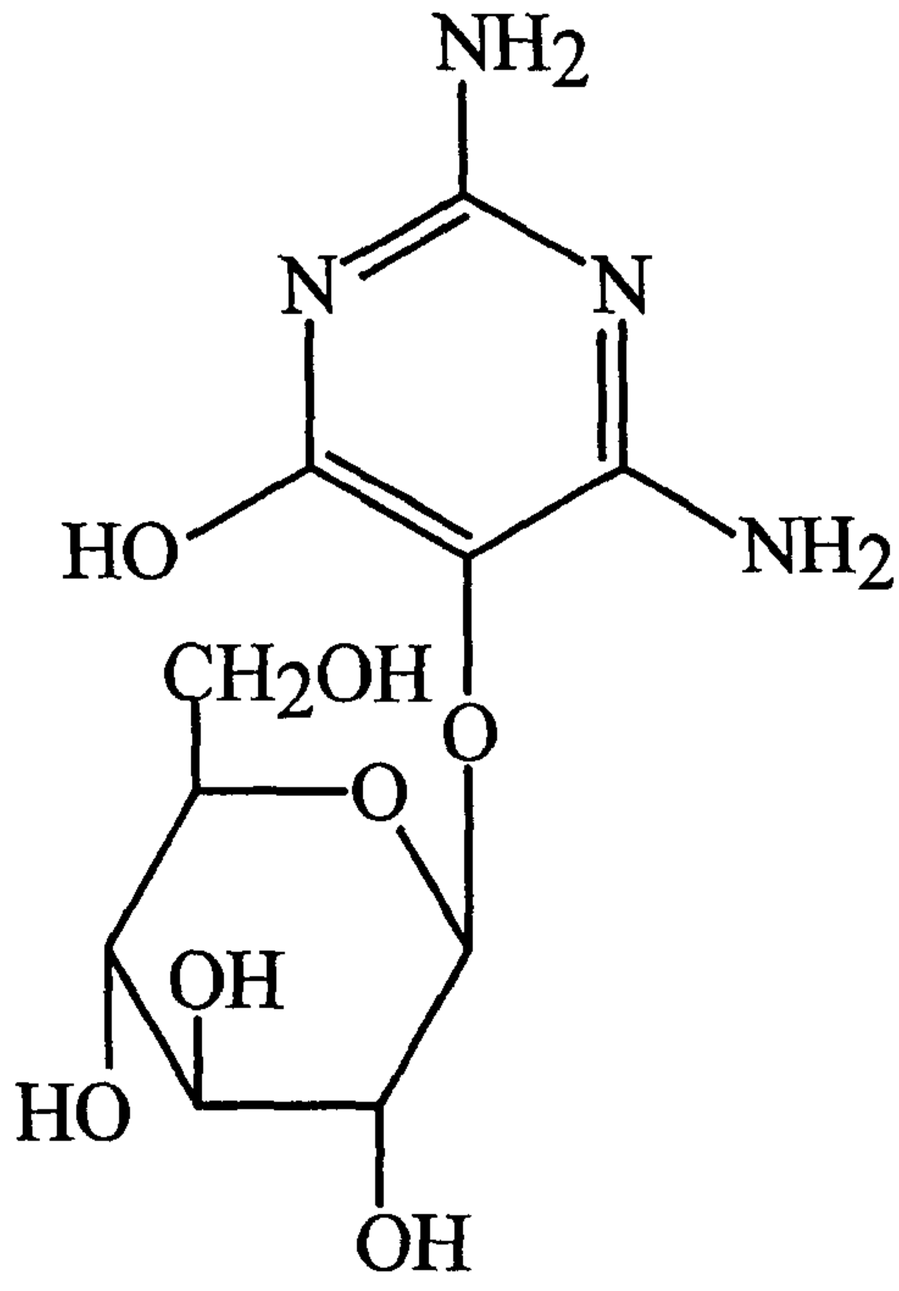


Figure 1.20: Vicine, a putative hypoglycaemic compound from *Momordica charantia* seeds.

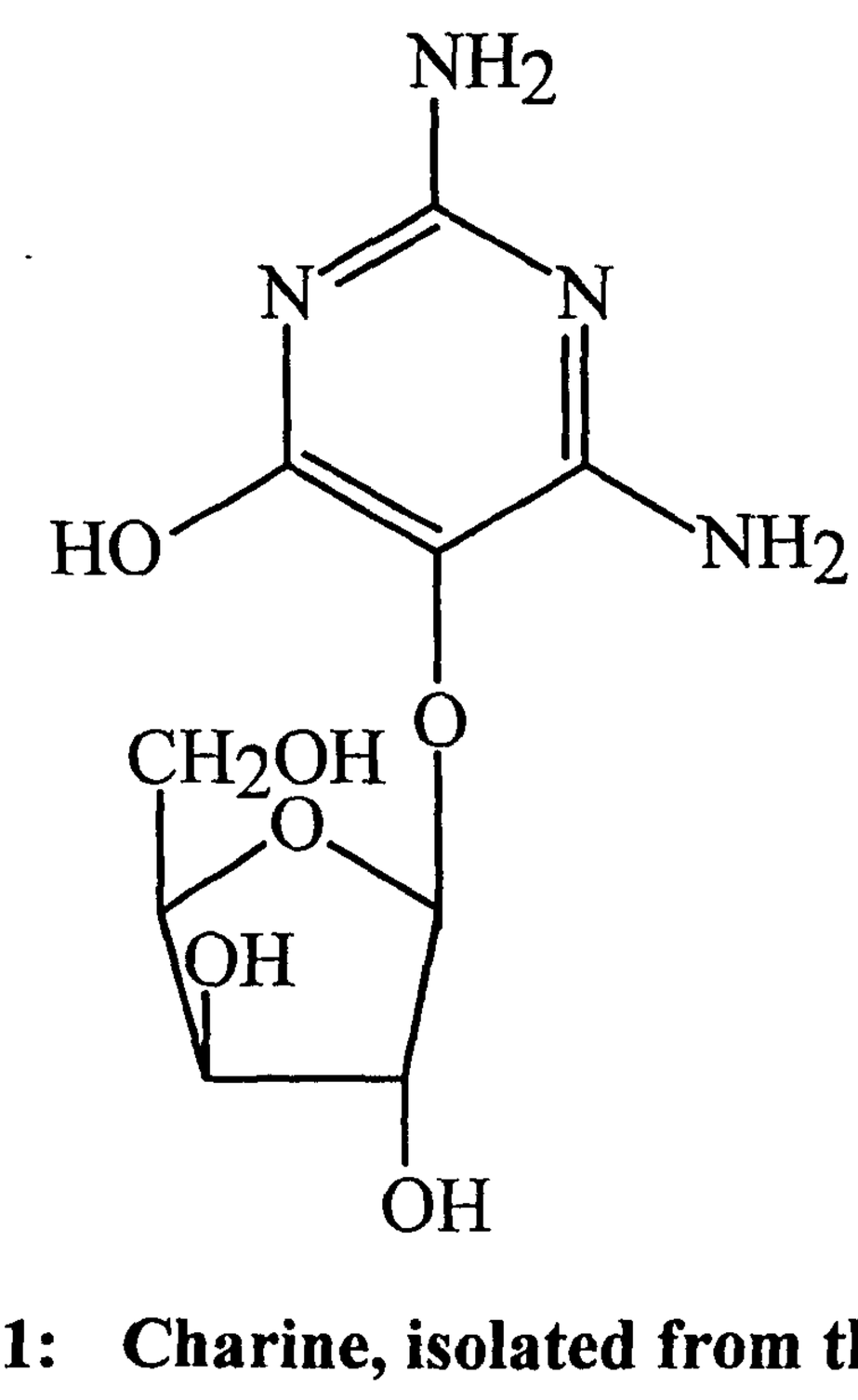


Figure 1.21: Charine, isolated from the unripe fruit of *Momordica charantia* L.

Table 1.6: Summary of the *in vitro* effects of karela fruit and seed extracts

Plant part	***Extract***	***Process studied***	***Effect***	***Reference***
Fruit	Ethanol, water	Glucose uptake into inverted gut	Inhibited	(1)
Fruit	Water	Insulin release from isolated islets	Stimulated	(2,3)
Fruit	Methanol (non-sapogenic)	Insulin release from isolated islets	Stimulated	(4,5,6)
Fruit	Juice	Gluconeogenesis in kidney slices	No effect	(7)
Fruit	Ethanol	Gluconeogenetic liver enzymes	Depressed*	(8)
Fruit	Ethanol	Glucose oxidation in liver	Inhibited	(1)
Fruit	Ethanol	Hexokinase (yeast)	Inhibited	(1)
Fruit	Ethanol	Hepatic glucose-6-phosphate dehydrogenase	Raised*	(8)
Fruit	Juice	Tissue respiration in diaphragm muscle	No effect	(7)
Fruit	Juice	Glucose uptake into diaphragm muscle	Stimulated	(7)
Fruit	Juice	Glycogen content in liver and muscle	Increased*	(7)
Fruit	Juice	Oxygen radical scavenging	Activity present	(9)

cont. →

Table 1.6 (continued): Summary of the *in vitro* effects of karela fruit and seed extracts

Plant part	*Extract*	*Process studied*	*Effect*	*Reference*
Fruit, seed	Ethanol, saline, water	Lipogenesis in adipocytes	Stimulated	(10,11)
Fruit, seed	Ethanol, saline, water	Hormone induced lipolysis in adipocytes	Inhibited	(10,12,13)
Seed	Proteins, low MW species	Lipogenesis in adipocytes	Stimulated	(11,14)
Seed	Lectins, saponin	Lipolysis in adipocytes	Inhibited	(12,13,15)
Fruit	Juice	Triglyceride content in adipocytes	No effect	(7)
Seed	Proteins	Adrenal steroidogenesis	No effect	(16)

* - Karela juice/extract administered *in vivo* prior to removal of tissue for analysis.

References: (1) Meir and Yaniv (1985); (2,3) Welihinda *et al.* (1982a,b); (4) Ali *et al.* (1993b); (5) Mosihuzzaman *et al.* (1994); (6) Rokeya *et al.* (1995); (7) Welihinda and Karunanayake (1986); (8) Shibib *et al.* (1993); (9) Rao (1991); (10,11) Ng *et al.* (1985, 1986a); (12,13) Wong *et al.* (1985a,b); (14,15,16) Ng *et al.* (1987a, 1986b, 1987b).

an indication of their mode of action.

Glucose absorption. A theoretical means by which glucose tolerance can be improved is by decreased absorption of glucose from the gut (Section 1.2). Meir and Yaniv (1985) reported that glucose uptake by inverted gut was inhibited in the presence of extracts of karela fruit. However, from the *in vivo* work of Day *et al.* (1990) and Higashino *et al.* (1992) it would appear that this may not be the mechanism involved in the action of karela since tolerance of intraperitoneally administered glucose is also improved. There have been no studies reported to date on effects of karela on enzymes involved in the digestion of carbohydrates, e.g. α-amylase.

Insulin secretion. In a number of *in vitro* studies (Welihinda *et al.*, 1982a,b; Ali *et al.*, 1993b; Mosihuzzaman *et al.*, 1994), extracts from the fruit have been found to stimulate insulin release from isolated pancreatic islet cells. The responsiveness of STZ- and alloxan- treated animals to karela, would seem to suggest that pancreatic stimulation is not involved. However, it must be noted that STZ and alloxan treatments may not result in complete destruction of pancreatic β-cells. For instance, in the study by Kedar and Chakrabarti (1982), STZ-treated animals were responsive to glibenclamide, which acts by stimulation of insulin release from the pancreas (Section 1.1.9(a)). In addition, Ali *et al.* (1993a) found that the effects of karela could be abolished by treating the animals with a higher dose of STZ. In some studies (Sharma *et al.*, 1960; Tiangda *et al.*, 1987) alloxan-treated rabbits were more responsive to karela extracts than normal animals. This may indicate a sensitisation of the β-cells to karela by alloxan. However, it should be noted that increased insulin levels *in vivo* have not been observed in karela treated mice (Day *et al.*, 1990), rats or humans (Leatherdale *et al.*, 1981).

Insulinomimetic effects. Karela juice shows certain insulinomimetic effects such as increased glucose uptake into muscle, stimulation of lipogenesis, and inhibition of lipolysis on tissue preparations *in vitro* (Table 1.6). *In vitro* tests on tissues taken from animals treated with karela have also shown a depression of hepatic gluconeogenetic enzymes, and increased liver and muscle glycogen.

There is conflicting data on effects of karela extracts on tissue respiration. Welihinda and Karunanayake (1986) found that karela juice did not show any effect on tissue respiration in diaphragm muscle *in vitro*. However, Meir and Yaniv (1985) reported that karela inhibited the oxidation of glucose by liver tissue, possibly at the first step in glycolysis, i.e. phosphorylation by hexokinase. These contradictory results may be due to differences in the tissues, methodology and type of karela preparation used. A more reliable indicator of effect of karela on tissue respiration may be that demonstrated by Shibib *et al.* (1993). Liver glucose-6-phosphate dehydrogenase (G-6-PDH) activity was elevated on *in vivo* administration of karela ethanolic extract by gastric intubation. This would enhance the utilisation of glucose by the liver leading to a lowering in blood glucose.

The lipogenic and anti-lipolytic effects of karela juice *in vitro* are shared by seed extracts. A saponin (not identified) and proteins have been found to account for the *in vitro* effects of the seeds. The proteins are believed to be lectins; the abortifacient proteins α- and β- momorcharins also found in the seeds are not active in this assay (Wong *et al.*, 1985a,b). However, against this, Welihinda and Karunanayake (1986) reported that adipose tissue of karela-treated rats did not differ significantly in triglyceride content from that of control animals.

Thus, inhibition of glucose absorption, insulin secretagogue activity and insulinomimetic effects have been attributed to karela in *in vitro* tests. However, not all of these have been fully supported by *in vivo* data, probably due to the compounds showing activity *in vitro* not being bioavailable *in vivo* (Section 1.2.5).

1.4.5 Phytochemicals isolated from Momordica charantia and their relationship to its anti-diabetic effects

Since the early 1960's, a number of phytochemicals have been isolated from *Momordica charantia* fruit, seeds and whole plants. A review of the known constituents was published in 1989 (Fiche Espèce, 1989). These data and supplementary information are given in Tables 1.7-1.9. In some cases, biological activities, such as insulinomimetic properties, protein synthesis inhibition, or insect attractant effects have been associated with the pure compounds or with fractions rich

Table 1.7: Phytochemicals isolated from *Momordica charantia* fruit

Phytochemicals	*Comments*	*References*
Steroids/Terpenoids/Cucurbitacins		
Charantin (Figure 1.19)	Mixture (1:1) of β-sitosterol and stigmastadienol glycosides.	Sucrow (1965,1966)
	Hypoglycaemic in rabbits on oral or intravenous administration.	Lotlikar and Rajarama Rao (1960/61; 1966)
	Does not stimulate insulin release from pancreatic cells *in vitro*.	Welihinda *et al.* (1982a)
Momordicosides (Figure 1.22)	G, F_1, F_2, I - non bitter cucurbitacin glycosides.	Okabe *et al.* (1982a,b)
	K, L - bitter cucurbitacin glycosides.	Okabe *et al.* (1982b,c)
Acylglucosylsterols	Antimutagenic against mitomycin C in mouse.	Guevara *et al.* (1989)
Linolenoylglucopyranosylclerosterol	Ripe fruit; attractant for *Dacus cucurbitae* insect.	Saito and Kato (1987a)
	3 pentacyclic triterpenes (momordicin, momordicinin and momordicilin) and a sterol (momordenol) isolated (Figure 1.24).	Begum *et al.* (1997)
Amino acids	Ripe fruit.	Dhalla *et al.* (1961)
Lipids		
Fatty acids	Most abundant (45%) is α-eleostearic acid.	Yuwai *et al.* (1991)
Galactopyranosyldilinolenoylglycerol	Attractant for *Dacus cucurbitae.*	Saito and Kato (1987b)
Phenolic compounds	12 phenolic acids and flavonoids reported.	Venkataramaiah and Rao (1983) cont.→

Table 1.7 (continued): Phytochemicals isolated from *Momordica charantia* fruit

Phytochemicals	*Comments*	*References*
Proteins		
P-insulin, V-insulin	11k Dalton; hypoglycaemic in man and animals (parenteral).	Khanna *et al.* (1981)
Acid ethanol fraction	Stimulates lipogenesis; inhibits lipolysis in adipocytes *in vitro*.	Ng *et al.* (1985)
Acid acetone powder	Inhibits lipolysis in adipocytes *in vitro*.	Wong *et al.* (1985b)
Guanylate cyclase inhibitor	Found only in ripe fruit; inhibits the enzyme in rat tissue.	Vesely *et al.* (1977)
	Inhibits growth of prostate adenocarcinoma.	Claflin *et al.* (1978)
Cytostatic factors (ripe fruit)	11k and 70k Dalton; cytostatic to leukaemic lymphocytes but not normal ones; inhibits RNA, DNA and protein synthesis.	Takemoto *et al.* (1982a,b)
Anti-lymphoma factor (ripe fruit)	40k Dalton; produces transferable resistance to lymphoma in mice.	Takemoto *et al.* (1984)
	Lymphocytes from treated mice more sensitive to concanavalin A.	Cunnick *et al.* (1984)
Nucleosides	Charine (pyrimidine arabinopyranoside; Figure 1.21)	El-Gengaihi *et al.* (1995)
Uncharacterised compounds		
Alkaloid fraction	Slow acting hypoglycaemic effect in fasted STZ rats.	Day *et al.* (1990)
Kakra 1b, 111a, 111b	Non-steroidal hypoglycaemic agents.	Srivastava *et al.* (1993)
Anionic substance	From ripe fruit; M. wt. about 150; inhibits tubulin polymerisation.	Fletcher *et al.* (1983)
Buffer extract	Inhibits tumour formation; possible immunostimulant.	Jilka *et al.* (1983)

Table 1.8: Phytochemicals isolated from *Momordica charantia* seeds

Phytochemicals	*Comments*	*References*
Proteins		
Galactose binding lectins	Lectins I and II (both 26k Dalton); only I is a haemagglutinin.	Li (1980)
	Momordica agglutinin (32k Dalton); haemagglutinin.	Lin *et al.* (1978)
	Momordica charantia lectin (MCL) 115k Dalton; inhibits protein synthesis & inactivates ribosomes.	Barbieri *et al.* (1979, 1980)
	Lectin of 120k Dalton size (MCL?); two binding sites and haemagglutination inhibited	Mazumder *et al.* (1981);
	by galactose and derivatives.	Khan *et al.* (1981)
	Lectin of 124k Dalton size (MCL?); insulinomimetic effects.	Ng *et al.* (1986c, 1987b)
Insulinomimetic extracts	Lectin (MCL?); antilipolytic in adipocytes.	Wong *et al.* (1985b)
	Saline extract; antilipolytic in adipocytes.	Wong *et al.* (1985a,b)
	Acid ethanol extracts; lipogenic and antilipolytic in adipocytes.	Ng *et al.* (1985, 1986a)
	Insulinomimetic peptides (8k Dalton).	Ng *et al.* (1987a)
Ribosome inactivators	Momordins	
	Tumour protein synthesis inhibitor (24k Dalton); haemagglutinin.	Lin *et al.* (1978)
	Momordins a and b (28k Dalton) isolated.	Minami *et al.* (1992)
	Amino acid sequence of momordins reported.	Minami and Funatsu (1993)
	Momordica charantia inhibitor (MCI)	
	Protein synthesis inhibitor (23k Dalton); not haemagglutinin.	Barbieri *et al.* (1980)
	Glycoprotein with pI 8.60.	Falasca *et al.* (1982)
	Immunosuppressive.	Spreafico *et al.* (1983)
	Known site of interaction with eukaryotic rRNA.	Endo *et al.* (1988)
	Momorcharins {α (29k Dalton); β (28k Dalton); γ (11.5k Dalton)}	Fong *et al.*(1996);
		Pu *et al.* (1996) cont. →

Table 1.8 (continued): Phytochemicals isolated from *Momordica charantia* seeds

Phytochemicals	***Comments***	***References***
Proteins		
Abortifacients	Saline extract abortifacient fraction AP II	
	Terminates pregnancy in rats; trophoblasts necrosed.	Shum *et al.* (1985)
	Non-haemagglutinins; no antilipolytic effects in adipocytes.	Wong *et al.* (1985a)
	Momorcharins (α, β)	
	α (29k Dalton) and β (28k Dalton) forms equipotent in mice.	Yeung *et al.* (1986)
	β inhibits growth of mouse embryos and endometrial cells.	Chan *et al.* (1985)
	Non-haemagglutinins; no antilipolytic effects in adipocytes.	Wong *et al.* (1985b)
	Immunosuppressants; tumour growth inhibitors; lyse DNA.	Go *et al.* (1992)
	Toxic to hepatocytes *in vitro*.	Ng *et al.* (1994)
Antivirals	MCI inhibits multiplication of herpes simplex virus I.	Foa-Tomasi *et al.* (1982)
	Momorcharins (α, β) inhibit HIV replication.	Lifson *et al.* (1988)
	MAP 30 (30k Dalton) inhibits HIV infection and replication.	Lee-Huang *et al.* (1990)
Miscellaneous	2 storage proteins (4 × 5.5k Dalton subunits).	Li (1977)
	Anticancer fractions CAP-II; inhibits sarcoma growth in mice.	Pan *et al.* (1985)
	Ribonuclease Mc (21k Dalton) differs from fungal RNAses.	Ide *et al.* (1991)
	MCTI (*Momordica charantia* trypsin inhibitors) A, B, C (30k Dalton).	Huang *et al.* (1992)
	MCTI-I, MCTI-II and MCEI (*Momordica charantia* elastase inhibitors) I.	Hara *et al.* (1989)
	MCTI-III and MCEI-II, III, IV.	Hamato *et al.* (1995)
	Napin-like proteins; 1:1 disulfide-linked complexes of a small chain (MS1,2,3 or 4) & a large chain (ML).	Neumann *et al.* (1996)

cont.→

Table 1.8 (continued): Phytochemicals isolated from *Momordica charantia* seeds

Phytochemicals	***Comments***	***References***
Nucleosides		
Vicine (Figure 1.20)	Isolated from seeds; may be linked to favism.	Dutta *et al.* (1981); Barron *et al.* (1982)
	Hypoglycaemic in rats; non-haemolytic to sheep erythrocytes.	Handa *et al.* (1990)
Zeatin + riboside	Cytokinins found in immature seeds.	Iyer *et al.* (1981)
Amino acids	15 amino acids reported including γ-amino butyric acid.	Barron *et al.* (1982)
Fatty acids	6 fatty acids reported including eleostearic acid.	Lakshminarayana *et al.* (1982); Chang *et al.* (1996)
Terpenoids	15 sterols and 3 pentacyclic triterpenoids from two seed varieties.	Ishikawa *et al.* (1986); Kikuchi *et al.* (1986)
Steroidal/Cucurbitacin glycosides		
Saponin fraction	Haemolytic; inhibits hormone induced lipolysis in adipocytes.	Wong *et al.* (1985b)
	Inhibits glucose incorporation into lipids in adipocytes.	Ng *et al.* (1986b)
Momordicosides (Figure 1.22)	A and B.	Okabe *et al.* (1980)
	C, D and E.	Miyahara *et al.* (1981)

Table 1.9: Phytochemicals isolated from *Momordica charantia* whole plants, vines or leaves

Phytochemicals	*Comments*	*References*
Steroids/Terpenoids/Cucurbitacins		
Saponin	Uncharacterised; non-haemolytic; from whole plant.	Rivera (1941)
Sterols and steroidal/cucurbitacin glycosides	Stigmasterol; stigmastadienol and stigmastadienol glucoside from leaves.	Ulubelen and Sankawa (1979)
	Cucurbita-5,24-dienol (plant part not stated)	Fiche Espèce (1989)
	Sitosterol and sitosterol glucoside from aerial parts of plant; not anti-convulsant or anti-inflammatory.	Lal *et al.* (1990)
	Momordicines I, II and III (Figure 1.23); bitter glycosides from leaves.	Yasuda *et al.* (1984)
	Momordicine II feeding deterrent to red pumpkin beetles.	Chandravadana (1987)
	3 Cucurbitane triterpenoids (not momordicines) from leaves (Figure 1.25).	Fatope *et al.* (1990)
Alkaloids (uncharacterised)	From whole plant; white precipitate with Mayer's reagent.	Rivera (1941)
	From whole plant; dose dependent anti-inflammatory effect.	Lal *et al.* (1990)
	Tertiary alkaloids in alcohol extract of leaves.	Ulubelen and Sankawa (1979)
Amino acids	γ-Amino butyric acid; may be hypotensive principle.	Durand *et al.* (1962)
Proteins	Guanylate cyclase inhibitor from leaves (and ripe fruit).	Vesely *et al.* (1977)
Long-chain compounds	Hentriacontanol.	Lal *et al.* (1990)
	Triacontanol; n-octosan; phytosphingosine.	Ulubelen and Sankawa (1979)

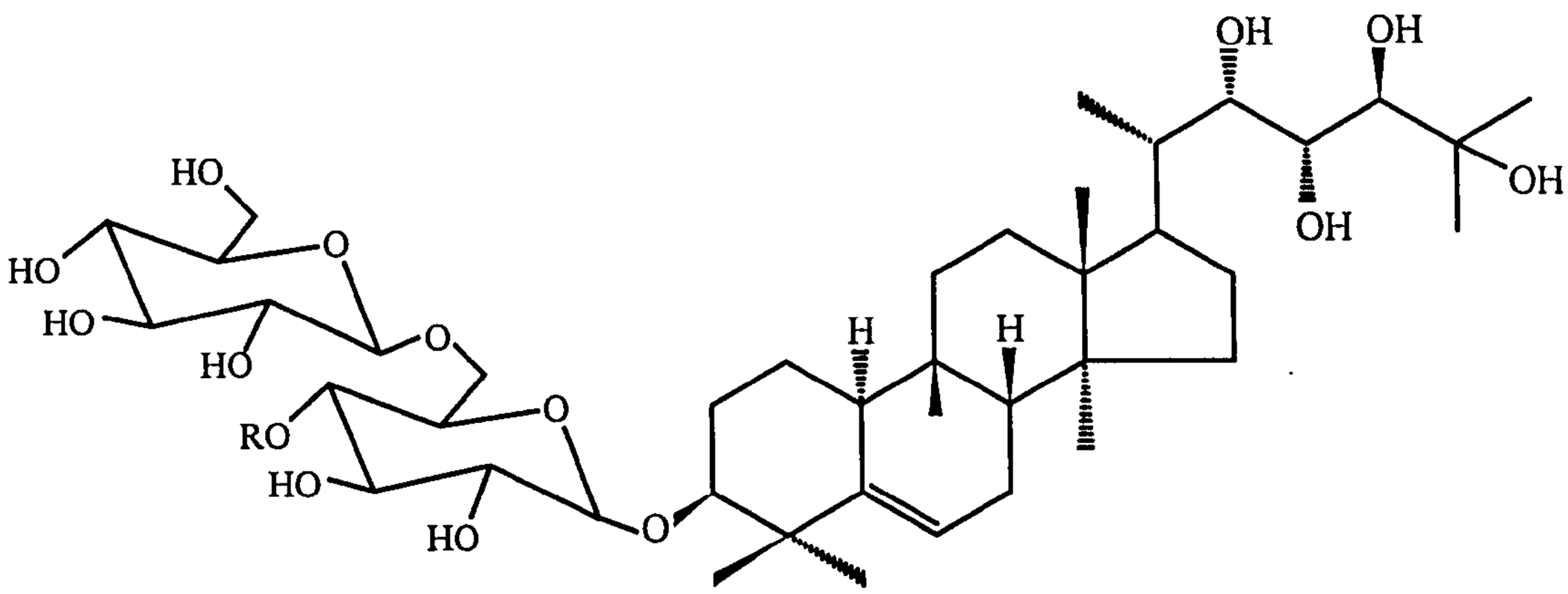
in a particular type of compound e.g. saponins or proteins. Possible identities which emerge for the hypoglycaemic principle in *Momordica charantia* are steroidal glycosides, insulinomimetic lectins and alkaloids; the evidence relating to each of these is discussed below.

Steroidal/cucurbitacin glycosides, steroids and terpenoids. The earliest reported active constituent of karela fruit was "Charantin" (Fig. 1.19), a mixture of glucosides of β-sitosterol and 5,25 stigmastadien-3β-ol (Lotlikar and Rajarama Rao, 1960/61, 1966). However, it is important to note that the dose of charantin required to elicit a hypoglycaemic response in rabbits was equivalent to 180 to 315g of fruit orally and 81g fruit intravenously, whereas a hypoglycaemic effect can be seen in rabbits with about 10 to 15g of the fruit per kg body weight.

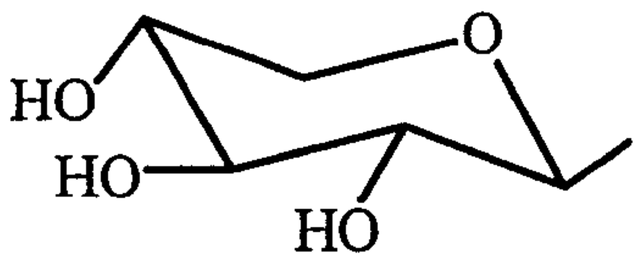
In 1975, Olaniyi isolated a substance "foetidin", from the whole plant of *Momordica foetida*, which was found to be identical in composition to charantin. Marquis *et al.* (1977) claimed that at 18 hours from administration, foetidin lowered blood glucose in fasting rats in an effect comparable to insulin. This claim is often quoted in the literature as support that the steroidal mixture is the active principle of *Momordica charantia*. However, a closer examination of the original data presented in the paper shows that foetidin was not significantly different from control at time points other than the 18 hour sample.

It is now known that *Momordica charantia* fruit, seeds and vines contain other cucurbitacin glycosides i.e. momordicosides and momordicines (Fig. 1.22, 1.23); Tables 1.7-1.9). Recently, three pentacyclic triterpenes (momordicin, momordicinin and momordicilin) and a sterol (momordenol) have been isolated from karela fruits (Begum *et al.*, 1997; Fig. 1.24) but no pharmacological studies have been reported so far on these compounds. A saponin fraction from the seeds of karela showed insulinomimetic effects *in vitro* (Wong *et al.*, 1985b; Ng *et al.*, 1986b). The contribution of steroidal constituents other than charantin to the *in vivo* anti-diabetic effects of *Momordica charantia* has not been evaluated.

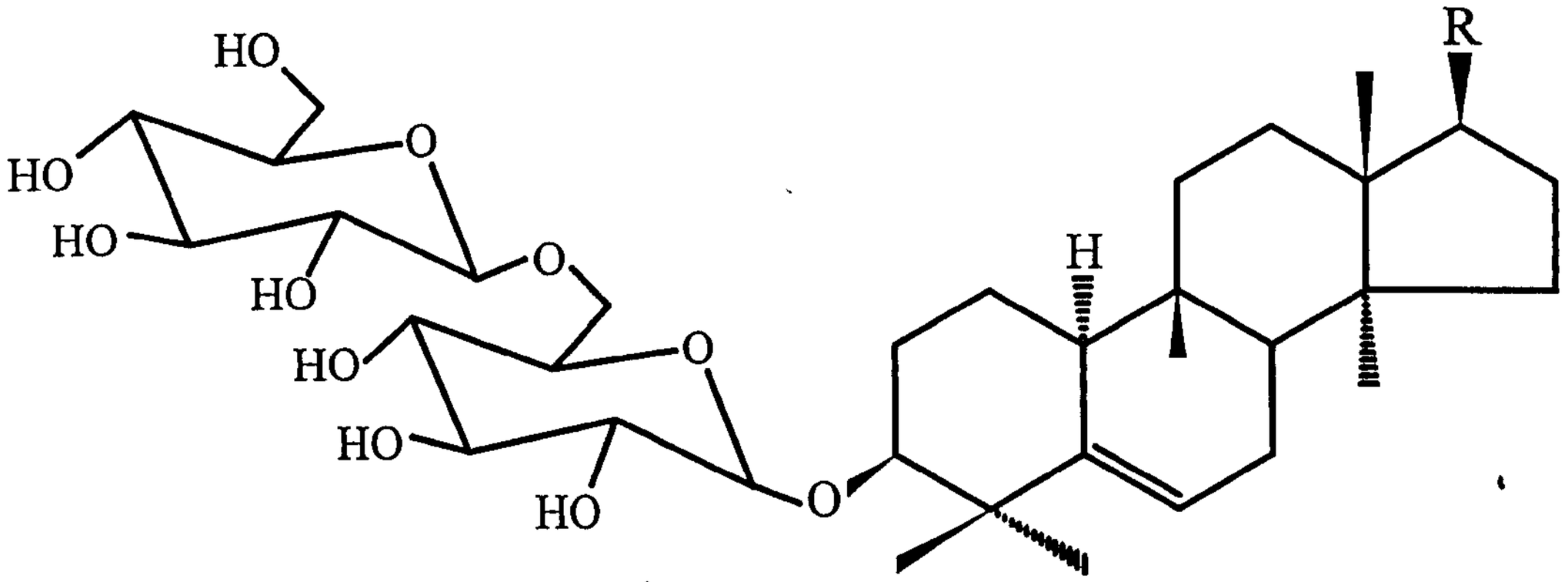
Insulinomimetic proteins. *In vitro* insulinomimetic effects have been observed with fruit proteins (Table 1.7) and seed proteins (Table 1.8). The active seed protein is



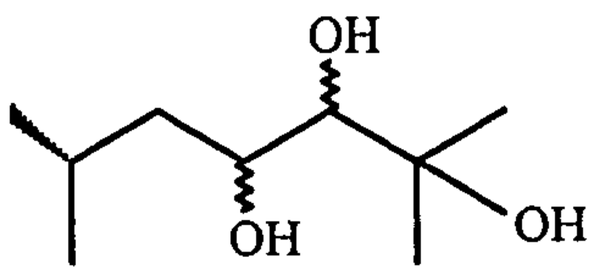
Momordicoside A R = H



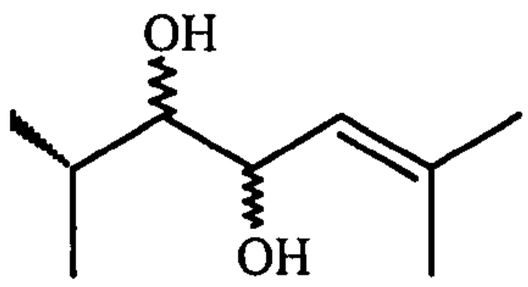
Momordicoside B R = Xylopyranosyl



Momordicoside C R =



Momordicoside D R =



Momordicoside E R =

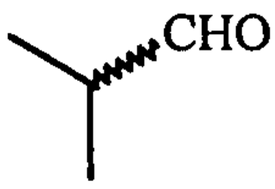
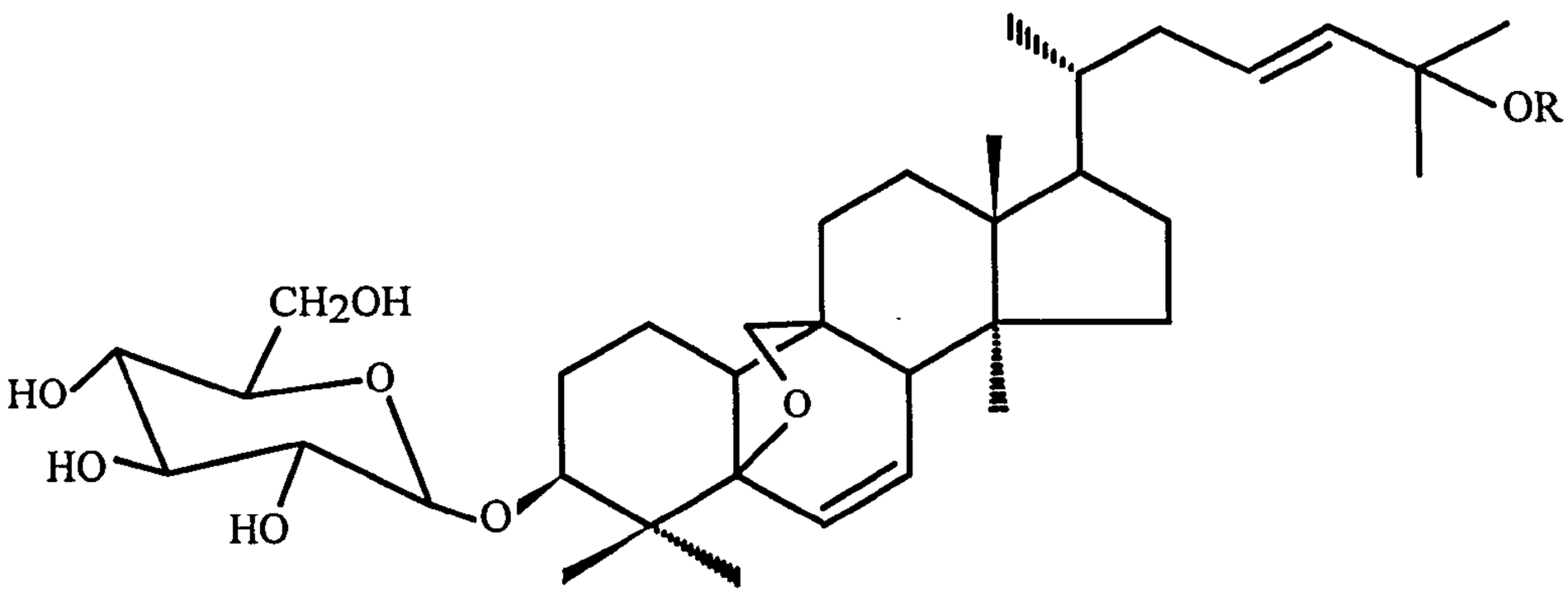


Figure 1.22: Momordicosides isolated from *Momordica charantia* fruit and seeds.

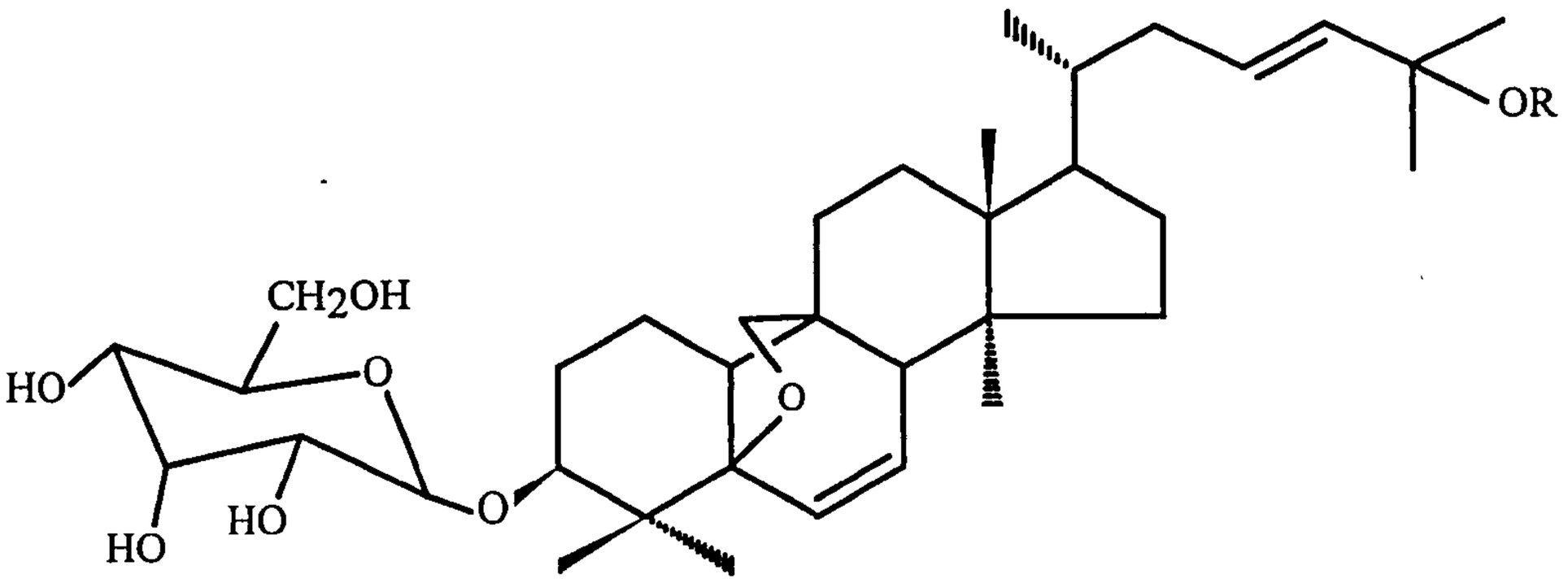
(cont.)



β-D-glucose

Momordicoside F_1 R = CH_3

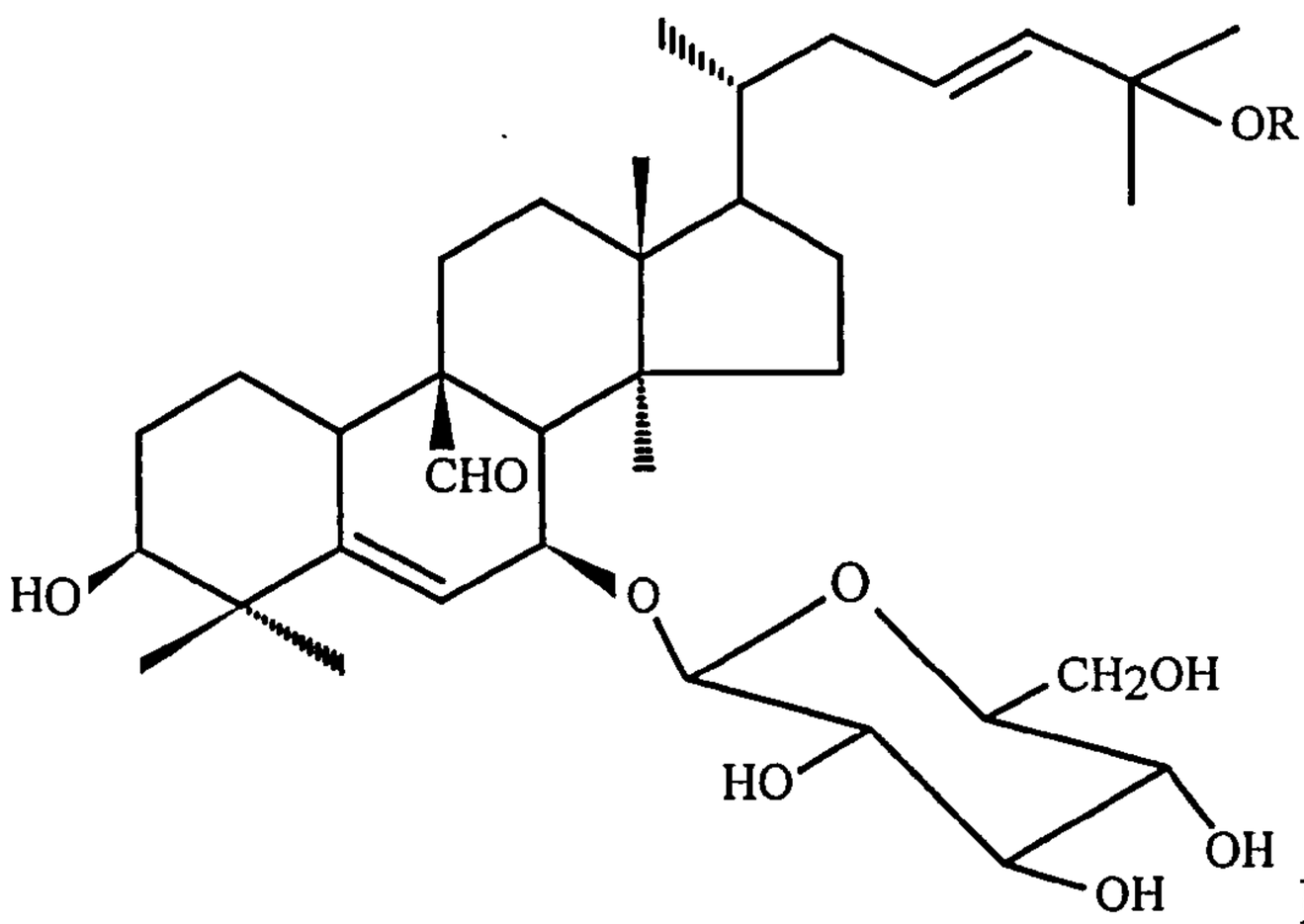
Momordicoside I R = H



β-D-allose

Momordicoside G R = CH_3

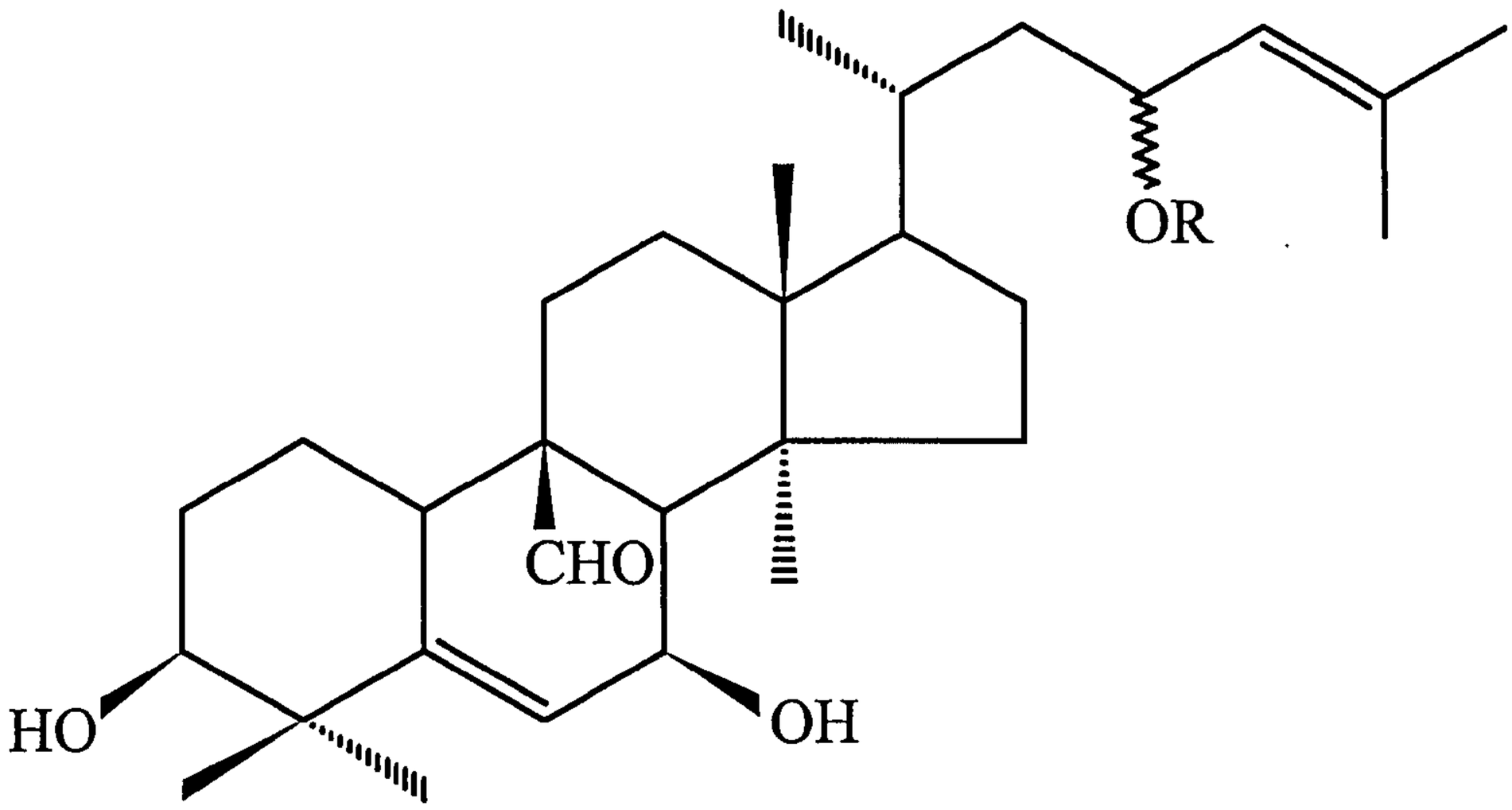
Momordicoside F_2 R = H



Momordicoside K R = CH_3

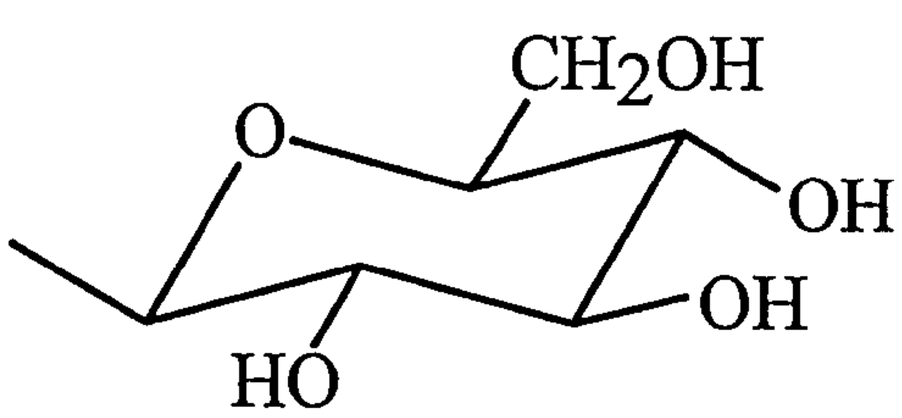
Momordicoside L R = H

Figure 1.22 (continued): Momordicosides isolated from *Momordica charantia* fruit and seeds.



Momordicine I R = H

Momordicine II R =



Momordicine III

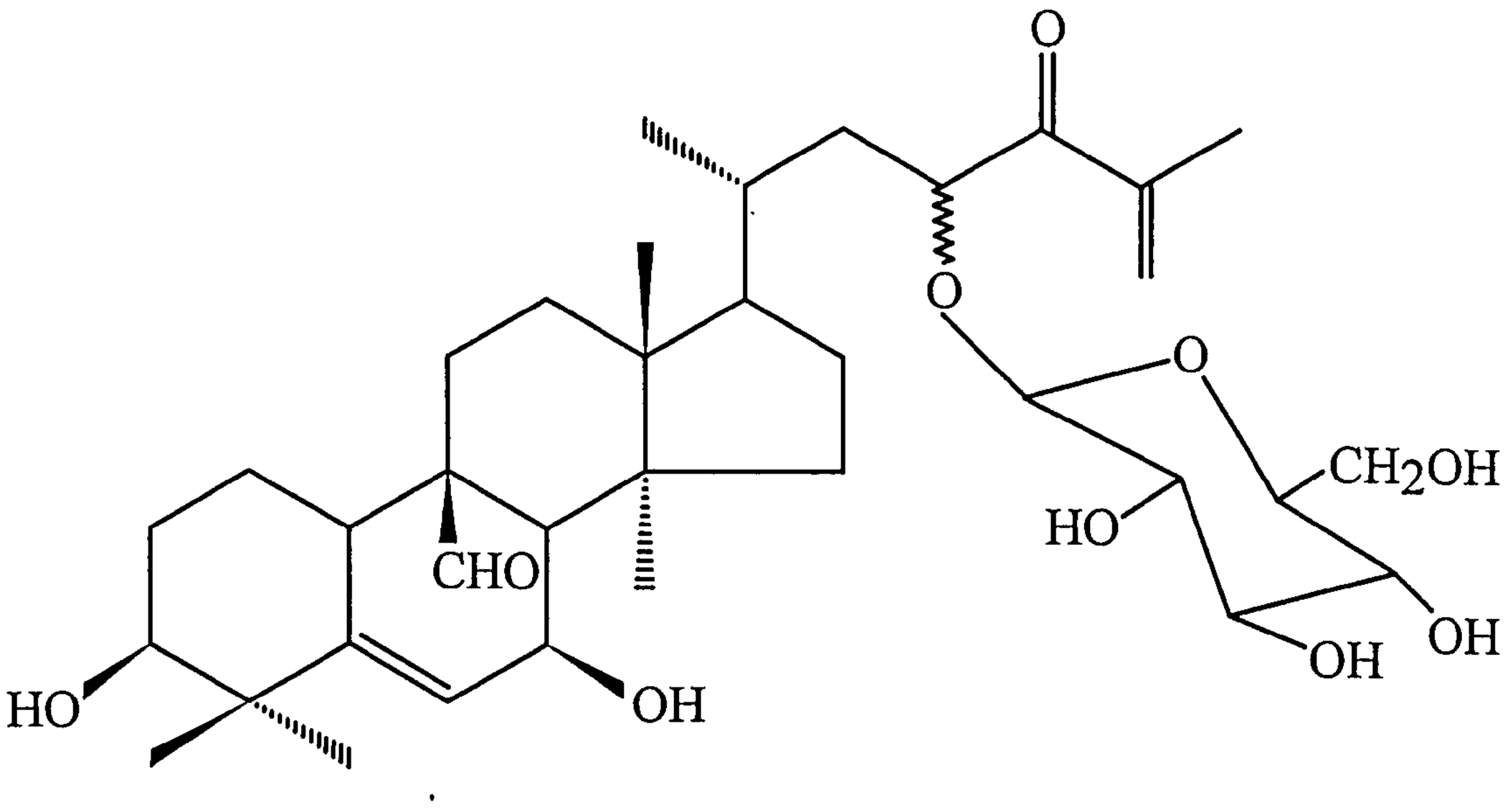
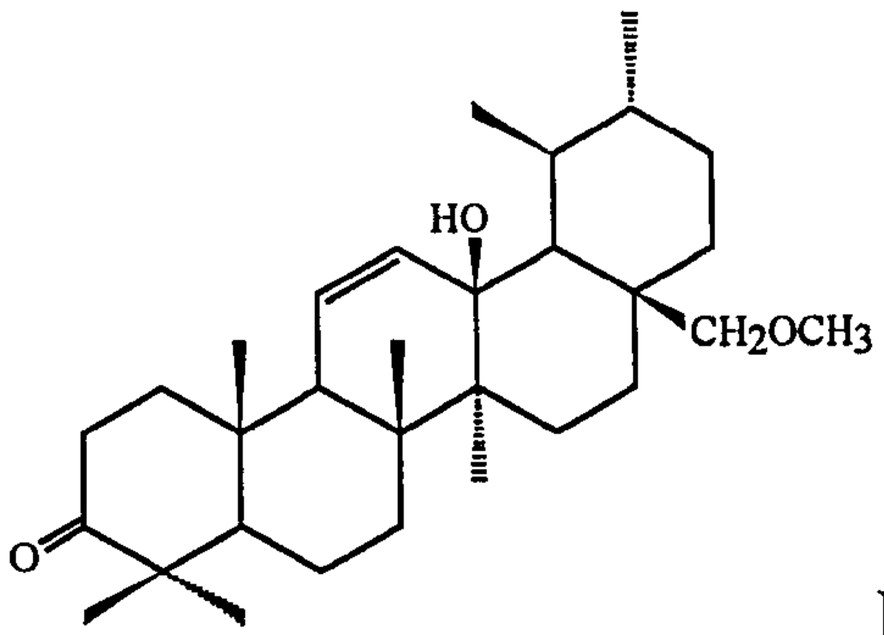
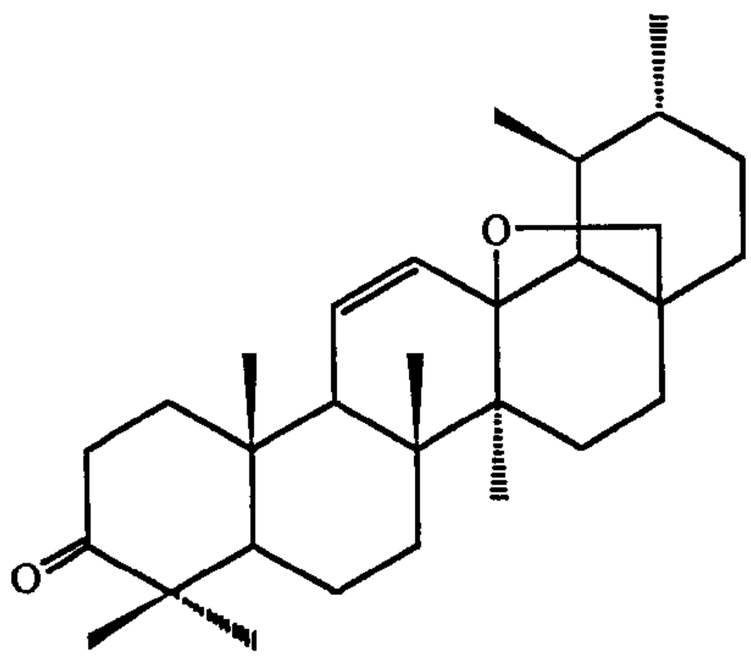


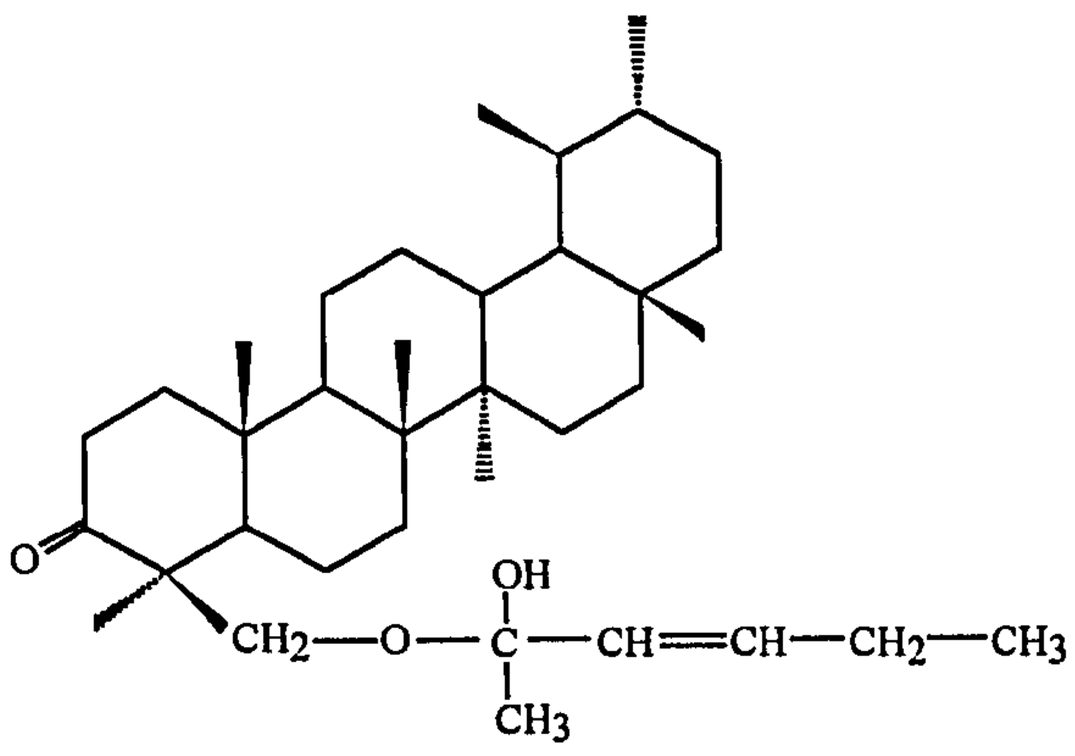
Figure 1.23: Momordicines isolated from *Momordica charantia* leaves



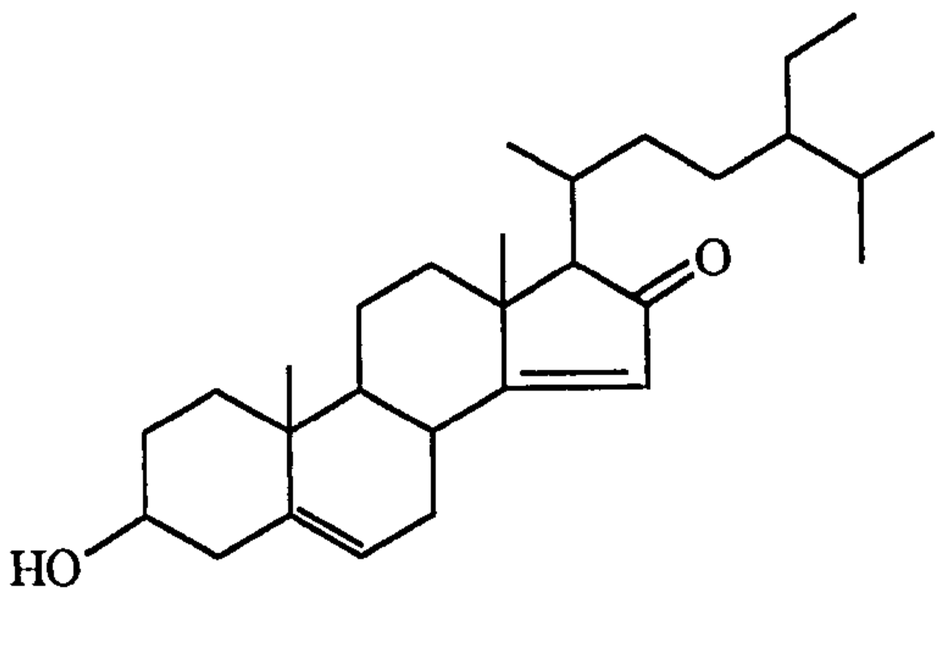
Momordicin



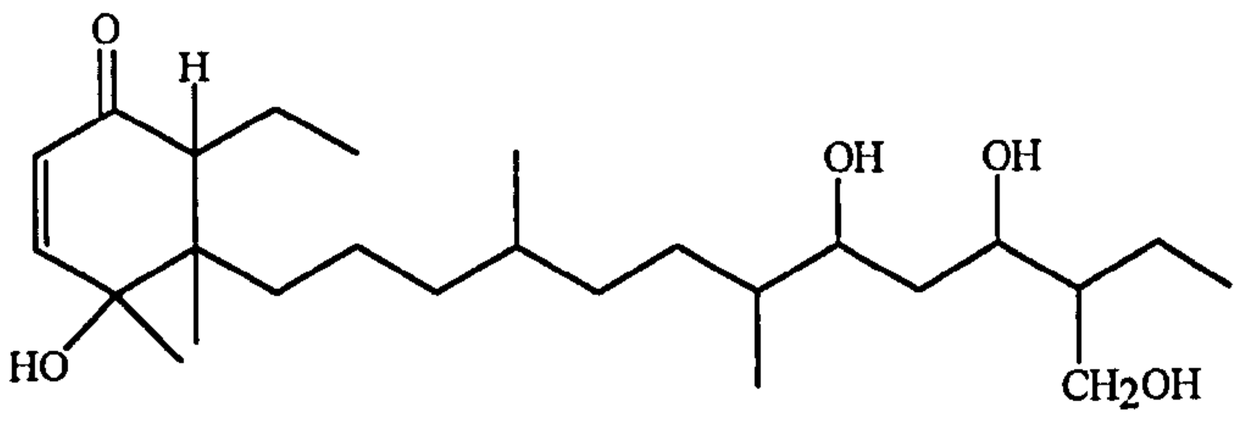
Momordicinin



Momordicilin

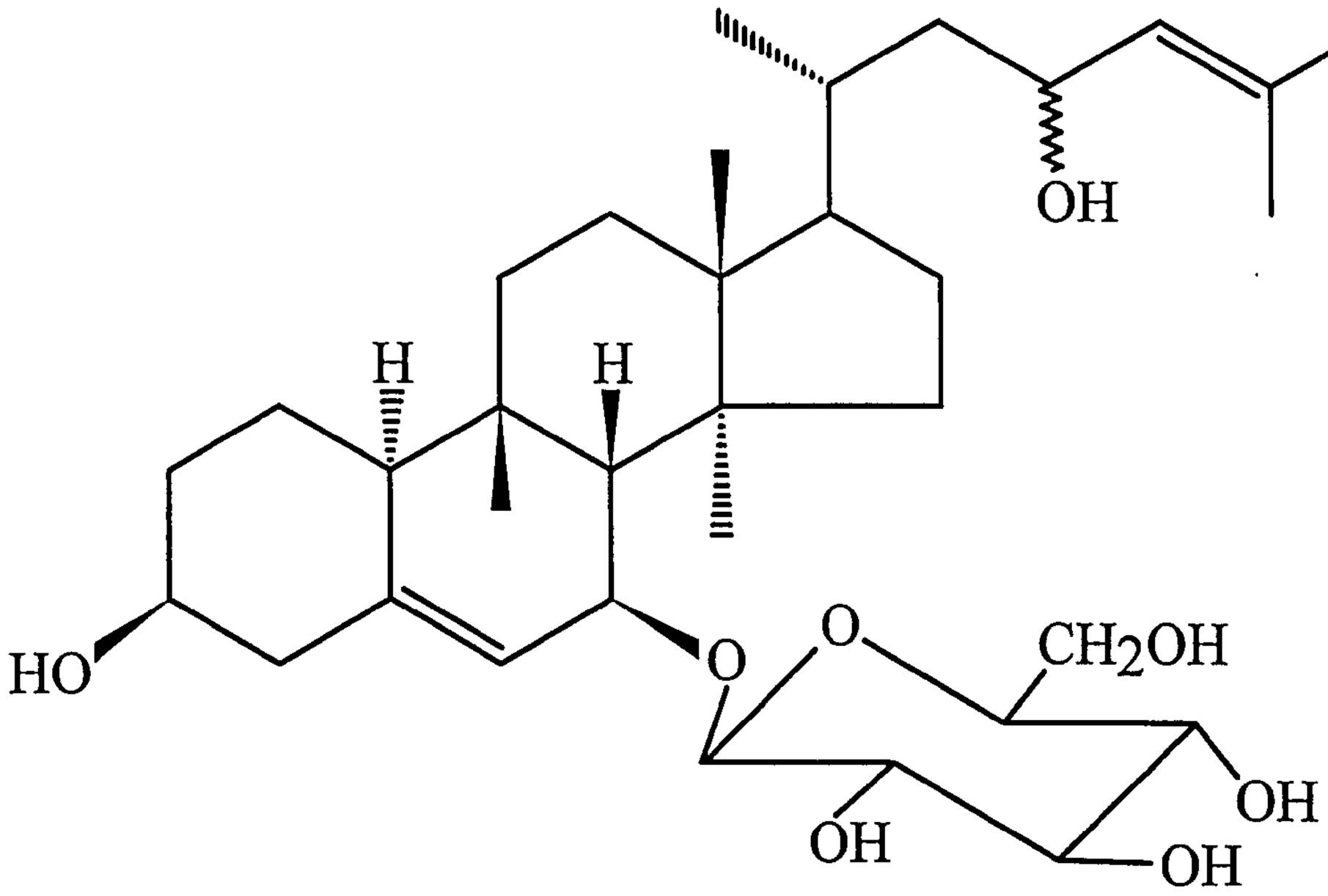


Momordenol

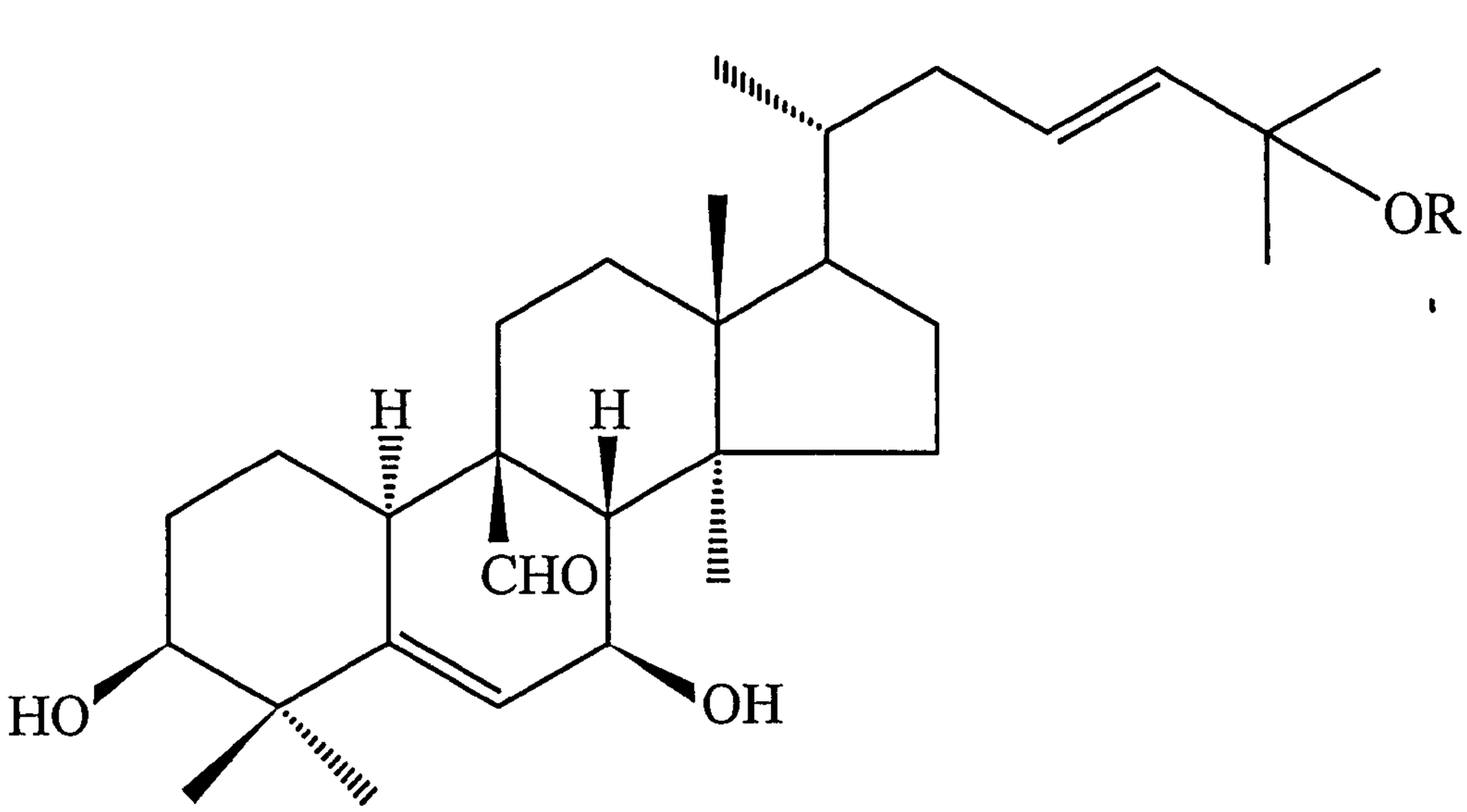


Momordol

Figure 1.24: New compounds isolated from *M. charantia* fruit (Begum *et al.*, 1997)



Triterpenoid 1



Triterpenoid 2 R = H

Triterpenoid 3 R = CH_3

Figure 1.25: Cucurbitane triterpenoids from *Momordica charantia* leaves

believed to be a galactose binding lectin (Table 1.8). Khanna *et al.* (1981) reported that an 11k Dalton protein (p-insulin or v-insulin) caused hypoglycaemia in man and laboratory animals on parenteral administration.

Proteins are generally considered to be inactive when administered by the oral route, as they would undergo extensive digestion by proteolytic enzymes. Thus the possibility of a polypeptide being responsible for the hypoglycaemic effects of the fruit or seeds when given orally must be viewed with some scepticism. However, against this, there is some evidence (Pusztai, 1986) that lectins may be absorbed into the bloodstream from the gastro-intestinal tract. Khanna (1985) has stated without any supporting data that p-insulin is also effective orally. In fact, the oral hypoglycaemic effect of p-insulin in diabetic rats has been reported by Jiangxin *et al.* (1991), and further investigations are required to confirm the results (Section 1.4.3).

Alkaloids. Day *et al.* (1990) reported that hypoglycaemic activity of fractionated karela fruit juice resided in an alkaloid-rich fraction. The alkaloids have not been isolated or characterised. The pyrimidine nucleoside vicine (Fig. 1.20) has been isolated from the seeds (Dutta *et al.*, 1981; Barron *et al.*, 1982). This "alkaloid" has been found to induce hypoglycaemia in rats at an intraperitoneal dose equivalent to 16g of seeds per kg body weight (Handa *et al.*, 1990). Kedar and Chakrabarti (1982) found the seed powder to be orally effective in rabbits at 1-3g per kg body weight. Thus vicine may not account for all the activity of the seeds.

A pyrimidine arabinopyranoside named charine (Fig. 1.21) was isolated from the unripe fruits of *Momordica charantia* by El-Gengaihi *et al.* (1995). Since charine has a very similar chemical structure to vicine, it is therefore a potential hypoglycaemic compound in karela fruits and its hypoglycaemic activity remains to be investigated.

Kakra compounds. Srivastava *et al.* (1993) isolated three non-steroidal hypoglycaemic compounds (Kakra 1b, 111a and 111b) from the fruit which differ from earlier reported principles, i.e. p-insulin or charantin. The structure of these compounds had not been elucidated.

1.4.6 Other pharmacological and toxicological properties

A number of effects of *Momordica charantia* unrelated to diabetes have been investigated. No data are available on standard toxicity parameters e.g. LD_{50} values of the juice, seeds or plants. However some information on toxicity is available from observations made during experimental or clinical use of *Momordica charantia* extracts in animals or humans.

Anti-cancer. Protein fractions obtained from the fruit and seed of *Momordica charantia* have the ability to inhibit cell growth, guanylate cyclase activity and ribosomal activity (Tables 1.7 and 1.8). West *et al.* (1971) demonstrated inhibitory effects of whole plant extracts on seedling root growth, division of fertilised sea urchin eggs, rat foetal growth (if injected on day of mating) and the growth of Hep_2 cells in culture. They also reported a single case study of a leukaemia patient in whom regular intake of the extract led to a fall in white blood cell count, and an increase in blood haemoglobin.

Antiviral. The growth of herpes simplex virus I (Foa-Tomasi *et al.*, 1982) and human immunodeficiency virus I (Lifson *et al.*, 1988; Lee-Huang *et al.*, 1990) is inhibited by karela extracts. Increased T-cell count and a normalisation of the CD4/CD8 ratio seemed to occur in three HIV positive patients given regular doses of karela juice (Zhang, 1992b). The juice was administered as a retained enema i.e. rectally. This may explain its apparent effectiveness since the active anti-viral components of *Momordica charantia* are believed (Zhang, 1992b) to be the proteins α- and β-momorcharins and MAP 30 (Table 1.8), which would be expected to undergo hydrolysis by pancreatic enzymes if administered by the oral route. Furthermore, Bourinbaiar and Lee-Huang (1995) reported the enhancement of weak HIV antagonists, dexamethasone and indomethacin, by MAP 30, which suggested that use of MAP 30 in combination with low doses of dexamethasone and indomethacin may improve the efficacy of anti-HIV therapy.

Analgesic effects. A methanolic extract of the seeds from unripe fruit has been shown to produce a marked dose-dependent analgesic effect in mice and a much weaker effect in rats (Biswas *et al.*, 1991), but using different test systems for the two

species. Naloxone pretreatment failed to modify the analgesic response, suggesting that opioid receptors were not involved.

Anti-inflammatory effects. A dose related anti-inflammatory effect has been demonstrated using carageenin-induced rat hind-paw oedema (Lal *et al*., 1990). Free radical scavenging activity of the juice *in vitro* (Rao, 1991) may be involved.

Gastroprotective and ulcer healing activity. Karela fruit extract was shown to significantly inhibit the development of absolute ethanol and aspirin induced acute ulcers in laboratory rats (Fernandopulle *et al*., 1996).

Hypotensive action. "Cerasee" (aerial parts of *Momordica charantia*) extract showed a marked transient depressor effect on injection to the anaesthetised dog (Feng *et al*., 1962). Gamma amino butyric acid has been suggested to be responsible for this effect (Durand *et al*., 1962).

Antifertility effects. Oral administration of karela fruit extract (1.75g/day for 60 days) to male dogs resulted in testicular lesions and mass atrophy of spermatogenic elements (Dixit *et al*., 1978). Serum enzymes were normal implying that an infertility state was induced without altering general metabolic activity in the animal.

A study by Stepka *et al*. (1974) found that daily oral administration of the fresh juice of *Momordica* (species not stated) leaves to a group of female mice, reduced the fertility rate. This was reversed on withdrawal of the treatment. No pathological changes were seen in any of the maternal organs, but in some cases, concepti were seen as necrotic masses. In more recent work, proteins capable of inducing abortions (α- and β-momorcharins) and necrosis of placental trophoblasts have been isolated from *Momordica charantia* seeds (Table 1.8). It is possible that similar proteins occur in the leaves. Uterine bleeding has been induced in pregnant rats given karela juice (6ml/kg) orally (Zhang, 1992b), while 2 pregnant rabbits given karela juice (6ml/kg) suffered uterine haemorrhage and death within a few hours (Sharma *et al*., 1960). No such effect was noted in non-pregnant females.

Effects on growth, blood and serum lipids. Chronic administration of karela extract (1.75g orally per day for 20-60 days) to dogs resulted in elevated levels of serum cholesterol and non-esterified fatty acids, but no significant changes in body

weight or serum enzymes (Dixit *et al.*, 1978). Rats maintained on a diet containing freeze-dried karela for 8 weeks showed no change in food consumption rate or growth rate (Platel *et al.*, 1993). At the end of this period, organ weights (liver, kidney, testes, spleen, adrenals and heart) were similar to those of control animals. Blood cell counts, cell volume and haemoglobin parameters showed no significant difference to controls and remained within the normal range. However in this study, there was a significant decrease in blood cholesterol.

Hepatotoxicity. Following the administration of karela juice and seed extract to rats (10ml/kg body weight daily for 30 days), serum γ-glutamyl transferase and alkaline phosphatase was significantly elevated, but consistent histopathological defects were not observed in the liver (Tennekoon *et al.*, 1994). Therefore the elevated enzymes could either be due to mechanisms not obvious at the histological level or to enzyme induction. The prevalence of dilatation and/or congestion in the hepatic central veins and associated sinusoids was twice as high in the juice treated group as in the seed extract treated and control groups. Ng *et al.* (1994) have found that α- and β-momorcharins can induce cytoplasmic blebs and other morphological changes in rat hepatocytes *in vitro*. Secretion of various enzyme markers of cell damage is also raised.

Fatal doses in animals. Continuous single or twice daily oral administration of karela juice (6ml/kg body weight) to 6 rabbits resulted in 5 animals dying within 5-25 days (Sharma *et al.*, 1960). In an acute effect, pregnant but not normal rabbits, died within a few hours of receiving this dose (Sharma *et al.*, 1960). Rats given karela juice (18-40ml/kg body weight, by intraperitoneal route) became sluggish and died within 6-18 hours. Zhang (1992b) reported that pregnant rats died within a few hours of receiving karela juice (6ml/kg body weight) orally. In normal and alloxan diabetic rats given the same dose daily, 80-90% died within 5-23 days. Abdominal injection of the juice at (15ml/kg body weight) caused death in 6-18 hours. In rabbits receiving 10ml/kg orally per day, the majority were reported to have shown toxic effects, although the nature of these effects was not elaborated.

Toxicity in humans. Although toxicity has been observed in some animal studies, if extrapolated to humans, the relevance of the dose and route of administration must be considered. A dose of 6-10ml/kg would represent a dose of 400ml-1000ml for an adult. The normal adult dose is closer to 50ml, given orally. There are no published reports of fatal or serious effects in adults at this dose.

Patel *et al.* (1968) reported that administration of the juice or dried juice powder (equivalent to 250-500g of the fruit) to diabetic patients led to abdominal pain and diarrhoea. Zhang (1992b) has used orally or rectally administered fruit juice to treat HIV-positive patients. He reported that there was very low clinical toxicity. A patient who had been given the juice daily for over three years did not show any change in blood chemistry or any other untoward effect. Liver, kidney, heart or blood abnormalities have not been reported in any of Zhang's patients despite long term use of *Momordica charantia* fruit juice.

The only report of a potentially fatal reaction in humans is hypoglycaemic coma induced in two small children (Hulin *et al.*, 1988a, b). The children aged three and four required urgent medical attention following ingestion of a water extract of *Momordica charantia* leaves and vines. In both cases, the Sorrosi (cerasee) tea had been administered by their mothers early in the morning before any other food was consumed. Between 1-2 hours after ingestion, the children experienced convulsions followed by coma. Blood glucose was in the region of 1mM (normal range 3.8-5.5mM). Both patients recovered following hospital treatment.

1.4.7 Conclusion

The unripe fruit, seeds and aerial parts of *Momordica charantia* Linn. have been used as an anti-diabetic remedy in a number of areas of the world notably India, Sri Lanka, China and the West Indies. Limited studies on humans have shown that karela fruit juice reduces fasting blood glucose and improves glucose tolerance on acute administration. Prolonged administration causes a lowering of glycosylated haemoglobin in the blood, and decreases glycosuria and basal glycaemia. The hypoglycaemic and anti-hyperglycaemic effects of karela fruit and seeds have also been demonstrated in animal models. Through evidence from animal and *in vitro* studies, there is support for insulin secretagogue activity of the fruit. However,

enhanced insulin levels *in vivo* in response to administration of karela have not been observed. Insulinomimetic effects have also been observed *in vitro*, and there is evidence (Sarkar *et al.*, 1996) that hepatic glucose utilisation is increased *in vivo*.

It is interesting to note that variation in results occurred among studies on karela by different research groups. For example, some researchers reported the reduction in basal glycaemia after karela administration in normal and diabetic animal models (Akhtar *et al.*, 1981; Leatherdale *et al.*, 1981; Higashino *et al.*, 1992), while others did not observe this effect (Kulkarni and Gaitonde, 1962; Platel *et al.*, 1993; Platel and Srinivasan, 1995). These conflicting results may be due to variations in karela used, i.e. karela fruits from different geographical sources may vary in their chemical constituents and anti-diabetic activity (Lau *et al.*, 1996), due to subject variability, variations in dosages of karela administered, as well as differences in extraction methods employed in preparing karela extracts.

A wide range of compounds have been isolated from *Momordica charantia* fruit, seeds and vines, mainly steroidal or triterpenoid compounds and proteins. Suggested hypoglycaemic compounds include a polypeptide (p-insulin), a steroid mixture (charantin) and a pyrimidine nucleoside (vicine). However, none of these is fully supported as a sole active constituent by the scientific data available. It is possible that a number of active constituents with a range of biological effects beneficial to diabetes are present in the fruit.

Principal toxic properties of karela juice noted in animals are anti-fertility effects and hepatotoxicity, with death occurring on chronic oral treatment with doses of the order of 6ml/kg body weight. Pregnant females were particularly susceptible. Encouragingly, similar effects have not been reported in humans despite widespread use of the fruit juice both as a medicinal plant and as a vegetable.

Thus karela fruit or seeds may provide a new extract, compound or lead compound with anti-diabetic effects for the oral treatment of both IDDM and NIDDM patients. There is clearly a need for a thorough investigation of the orally active hypoglycaemic principles of *Momordica charantia*, in which due consideration is given to the dose and route of administration. The most logical approach would in the first instance be using a whole animal model (*in vivo*), with further studies carried out

on *in vitro* models to determine mode of action. Much scientific research has been conducted on *Momordica charantia* which supports its use in the treatment of diabetes. The next step would be to carry out a systematic study to isolate and identify the active component(s) of the plant, and elucidate their mode of action.

1.5 Aim of present study

"To use a systematic, relevant approach to isolate and identify orally active anti-diabetic phytochemicals in the unripe fruit of Momordica charantia, and to elucidate its mode of action."

As discussed in Section 1.4, numerous studies in humans, animals and *in vitro* models have demonstrated a potential beneficial effect of karela juice or extracts in diabetes. Although a number of hypoglycaemic principles have been proposed, either they have only been tested at relatively high doses compared to their concentrations in the fruit, or their effects on oral administration have never been fully investigated. In addition, the mode of action of karela has never been satisfactorily explained. Thus there is a need for further investigation of the oral hypoglycaemic activity of the unripe fruit of *Momordica charantia* to address these issues.

The aims of the present study are as follows:

- Despite the fact that a number of hypoglycaemic compounds have been proposed, none of these appear to totally satisfy the enquiry. Thus a systematic approach, an *in vivo* (Streptozotocin-induced diabetic [NIDDM] rat model) bioassay guided fractionation will be employed in the present investigation.
- The unripe fruit of *Momordica charantia* (karela) are found (either cultivated or wildly grown) in different parts of the world. Thus variations in physical appearance, chemical constituents and biological activity among different varieties of karela obtained from various geographical sources will be examined.
- Inconsistent results have been obtained from previous studies as to whether karela improves either glucose tolerance or basal glycaemia or both. Thus the effect of karela on glucose tolerance, as well as its effect on fasting glucose levels will be examined using a diabetic animal model.
- Information on the site of action of karela will be investigated using different routes of administration of glucose in glucose tolerance studies.

- So far, only *in vitro* but not *in vivo* insulin secretagogue activity of karela had been observed. Thus further investigation on plasma insulin levels *in vivo* in response to karela administration will be carried out.
- Some previous studies used parenteral route of administration of karela extracts or isolates. However, karela juice is known to exert an effect on oral administration. Due to the present project's main interest in isolating **orally active** anti-diabetic compounds from karela, only the oral route of administration of karela will be used in this study.
- *In vitro* models will be used, where appropriate, to examine the effect of karela extracts and isolates on particular mechanisms of action.

This research project was financially supported by an EPSRC Case Award (Lipha, France). The collaboration with the pharmaceutical company Lipha enabled all the *in vivo* studies (using a diabetic animal model) to be carried out (by the author, with the help of some technicians) in the Pharmacology research laboratory at Lipha, while all the phytochemistry work was carried out in the Pharmacognosy research laboratory at King's College London. Spectral analysis was provided by ULIRS AMX 400 Nuclear Magnetic Resonance Spectroscopy Service and ULIRS Mass Spectrometry Service at the Chemistry Department of King's College London.

Detailed experimental methodology and results obtained will be presented in the following chapters.

Chapter 2:

Botanical and Phytochemical studies

Chapter 2: Botanical and Phytochemical studies

2.1 Introduction

Many plants are found in various parts of the world as they grow equally well in numerous localities having similar climates and soils. In the case where the plant is of importance as food crop or medicinal use, it may even be largely cultivated rather than collected from the wild (happens in many countries). For example, the cinchonas, indigenous to the Andes of South America, were successfully introduced to Indonesia and India which at one time became the principal geographical sources for the bark and its quinoline alkaloids (quinine and quinidine for the treatment of malaria). However, it must be remembered that a plant may grow well in different situations but fail to produce the same chemical constituents. In the case of *Cinchona succirubra*, the plant only produces alkaloids if they are grown at altitude and not at low levels (Evans, 1989). Thus it is important to note that natural variation in chemical constituents may occur within the same plant species.

In this context, the plant *Momordica charantia* L. is found throughout the tropical countries. The plant either grows wild or is cultivated for use of the unripe fruit as a vegetable. This has led to the existence of a wide variety of cultivars. However, previous studies on *Momordica charantia* fruit (Section 1.4) seldom specified the geographical source or cultivar used.

No previous reports were found of comparisons between varieties. Thus the aim of the present study is to investigate both physical and chemical variations among different varieties of *Momordica charantia* (karela) fruit.

2.2 Plant materials

Seven varieties of fresh unripe karela fruit from different geographical sources were studied:

a) Karela fruit from Thailand (Fig. 2.1) - purchased from "Loon Fung Supermarket" in Chinatown, London, UK.

b) Karela fruit from Kenya, Africa (Fig. 2.2) - purchased from "The Food Centre", 70 Turnpike Lane, London, UK.

c) Karela fruit from Jaipur, India (Fig. 2.3) - purchased from market "Lal Kothi Sabji Mandi", Jaipur, by Dr Raka Kamal, Associate Professor of Botany at University of Rajasthan, Jaipur, India. The fruit were sent by courier and arrived slightly "off".
d) Karela fruit from Bombay, India (Fig. 2.4) - purchased from a street vegetable vendor in Bombay by Dr Amala Raman, Lecturer in Pharmacognosy, King's College London, UK.
e) Karela fruit from Nigeria, Africa (Fig. 2.5) - supplied by Dr Kemi Odukoya, Faculty of Pharmacy at University of Benin City, Benin, Nigeria.
f) Karela fruit from Bangladesh (Fig. 2.6) - purchased from a grocery store in Brixton market, London, UK.
g) Karela fruit grown in UK (Fig. 2.7) - purchased from a grocery store in Chinatown, London, UK.

All varieties were examined and authenticated by Dr Charles Jeffrey (Cucurbitaceae expert, Royal Botanic Gardens, Kew, UK).

2.3 Methods

2.3.1 Botanical studies

Among all different varieties of karela fruit obtained, the physical appearances such as colour, size (length and maximum width) and shape of the fruit were carefully examined and compared.

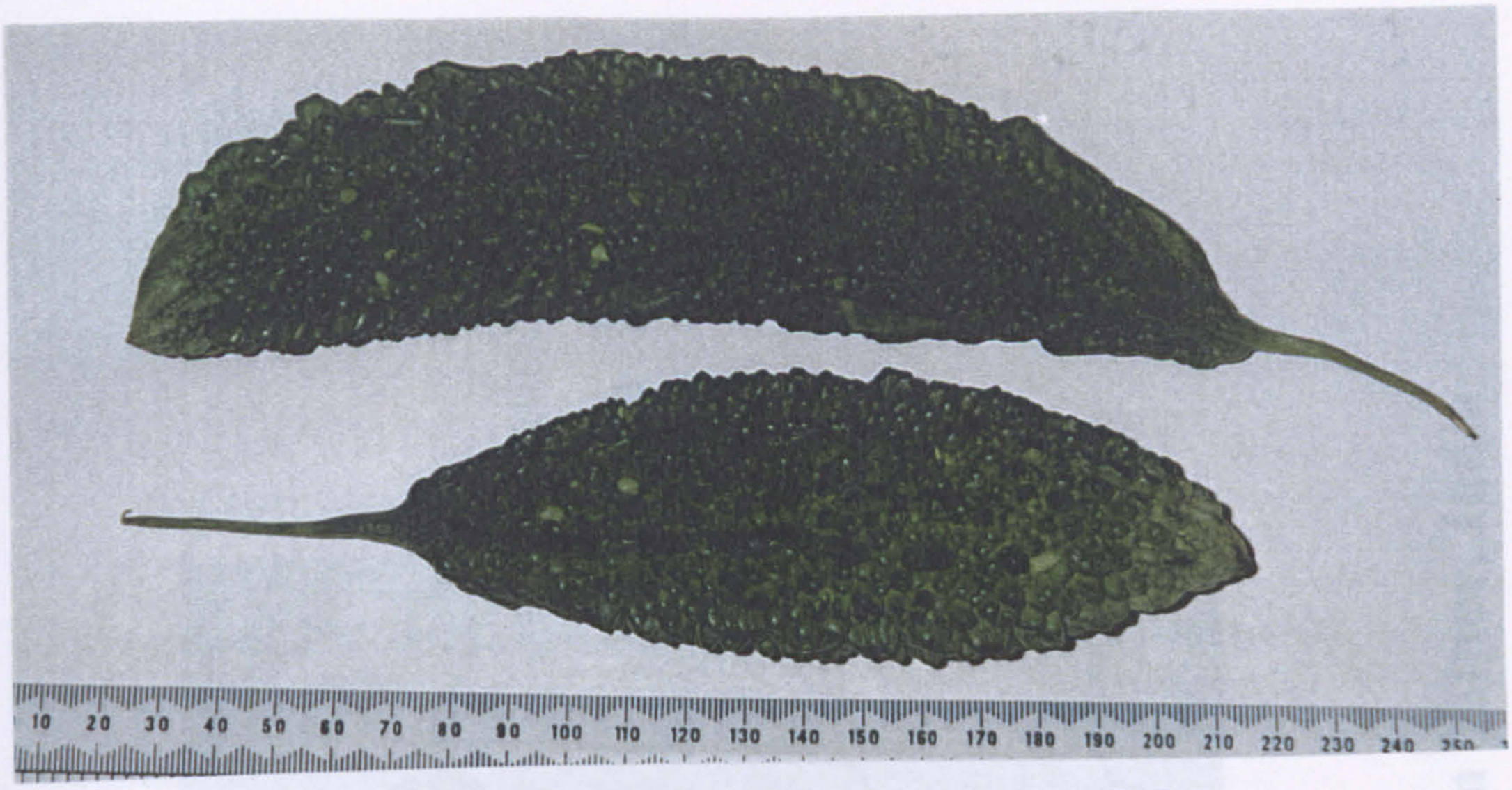
Seeds obtained from three varieties of karela fruit (Thai, Indian (Jaipur) and Kenyan) were planted in a greenhouse in Chelsea Physic Garden, UK. The seeds were sown in April and the plants were examined in September, after 5 months of growth. In addition, specimens of all three different varieties of *M. charantia* plants were collected and mounted as herbarium sheets. Herbarium voucher specimens (no. Mo10Hb1 (Thai), Mo10Hb2 (Kenyan) and Mo10Hb3 (Indian)) are deposited at the Pharmacognosy Section, Department of Pharmacy, King's College London.

2.3.2 Phytochemical studies

All different varieties of karela fruit, except those from India (Jaipur) and Nigeria (due to shortage of supply), were used for phytochemical studies.

length: 26-33 cm width: 6-7.5 cm

Figure 2.1: *Momordica charantia* fruit from Thailand



length: 11-27 cm width: 2.5-5.5 cm

Figure 2.2: *Momordica charantia* fruit from Kenya, Africa

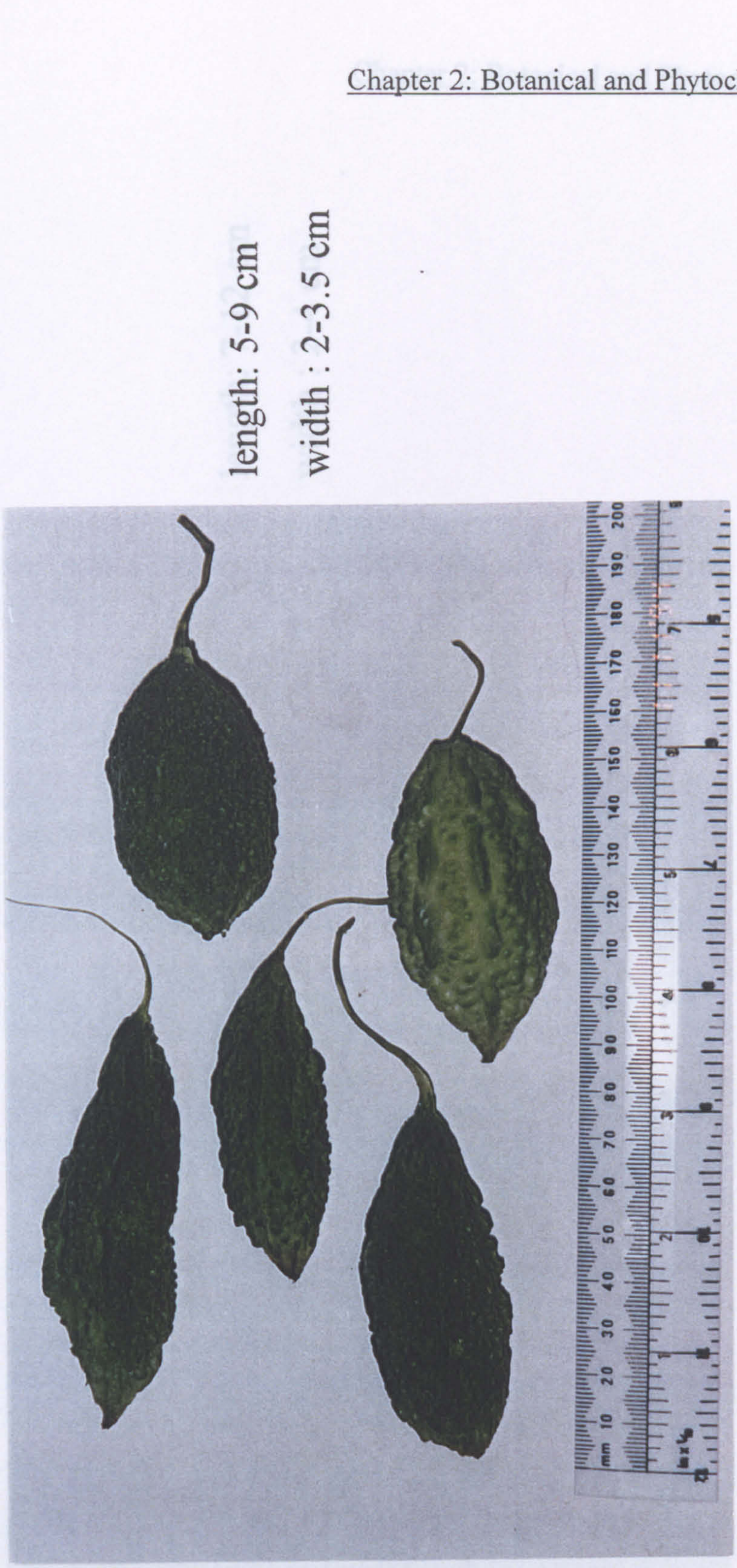
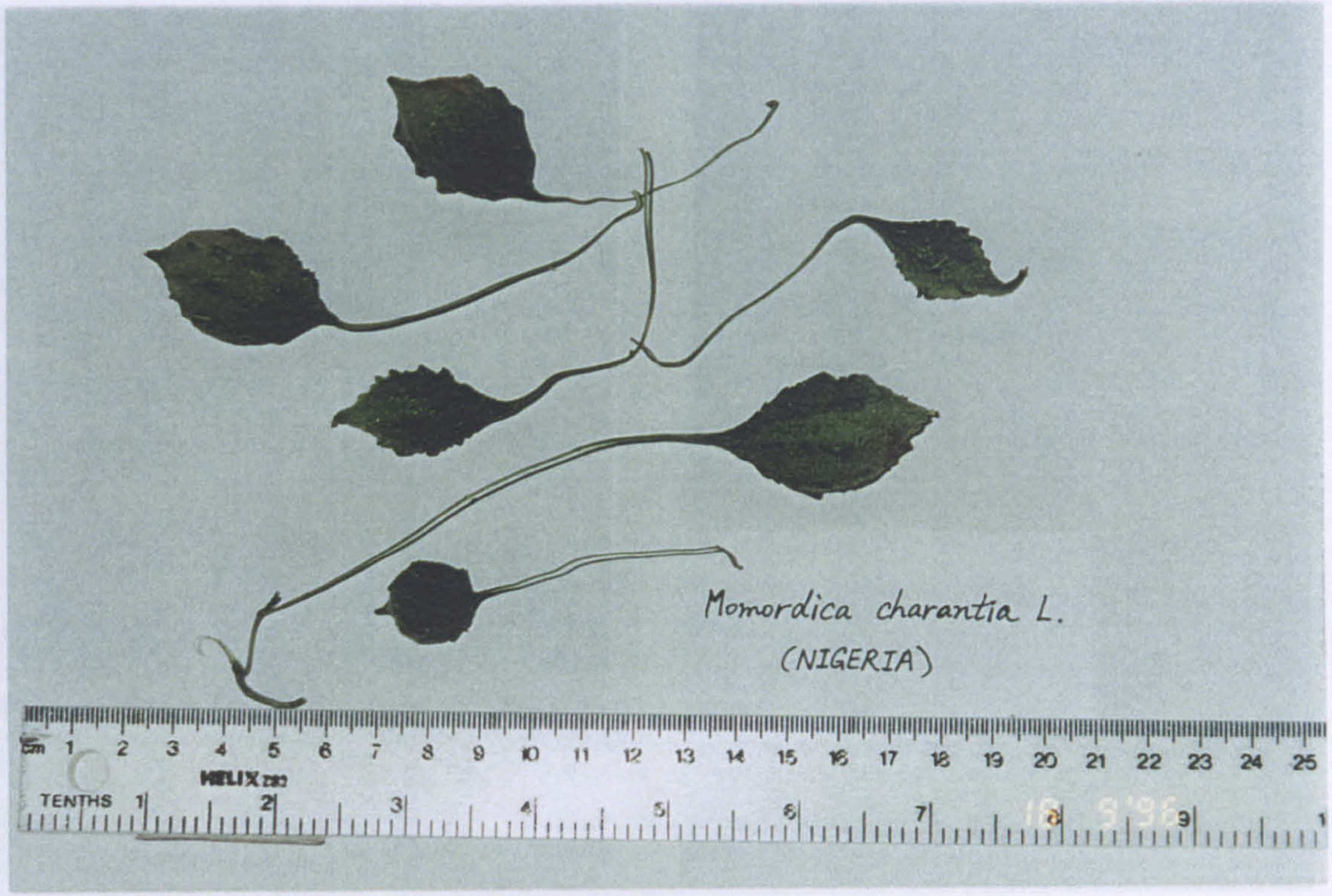


Figure 2.3: *Momordica charantia* fruit from Jaipur, India

length: 7-12 cm

width : 2-4 cm

Figure 2.4: *Momordica charantia* fruit from Bombay, India



length: 1-4 cm

width : 1-2.5 cm

Figure 2.5: *Momordica charantia* fruit from Nigeria, Africa

Figure 2.6: *Momordica charantia* fruit from Bangladesh

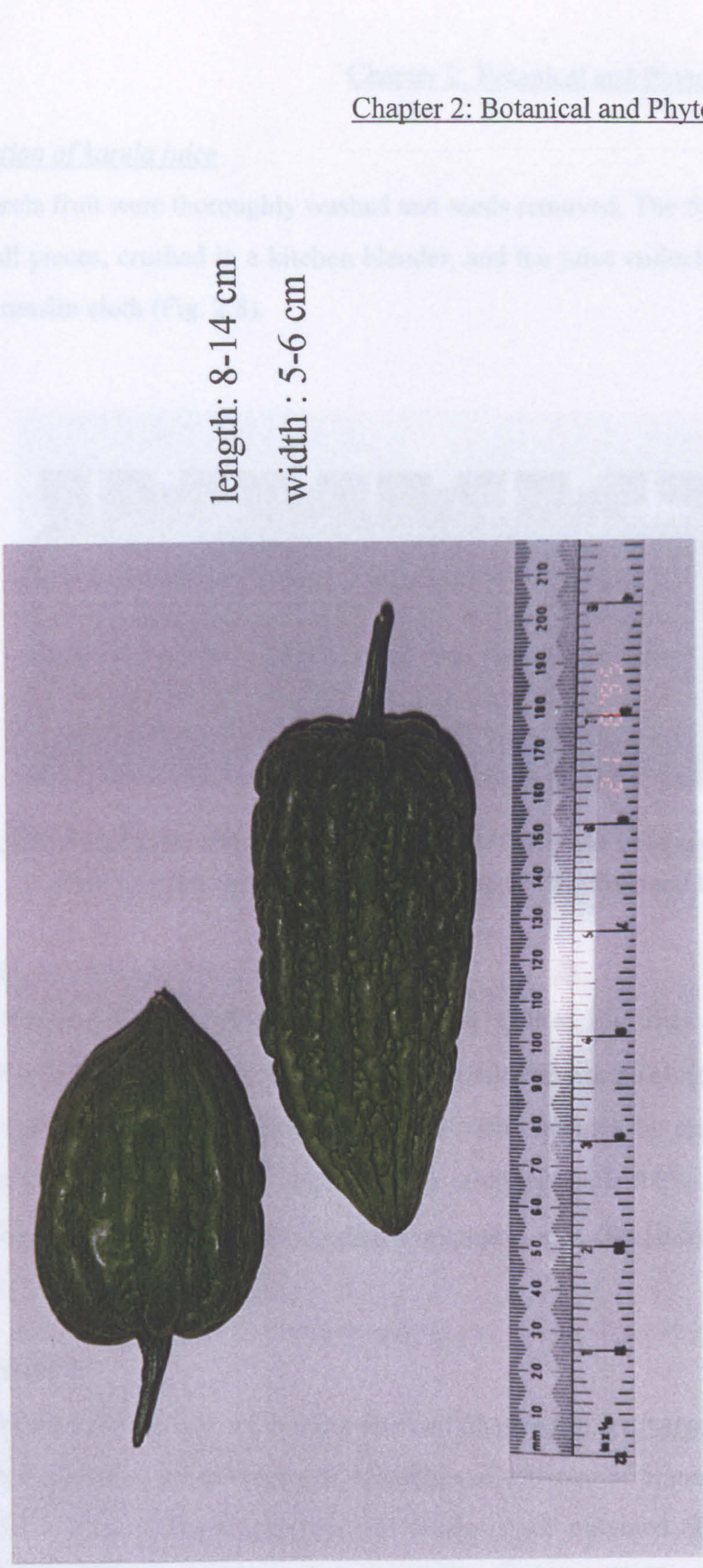


Figure 2.7: *Momordica charantia* fruit grown in England

Preparation of karela juice

Fresh karela fruit were thoroughly washed and seeds removed. The fruit flesh was cut into small pieces, crushed in a kitchen blender, and the juice collected after filtering through muslin cloth (Fig. 2.8).

Figure 2.8: Fruit juice obtained from karela of different geographical origins.
(Left to right: Thai, Bangladesh, UK, Indian (Bombay) and Kenyan)

Thin layer chromatography (TLC)

10µl of karela juice of different varieties were spotted on silica gel GF_{254} plate (Merck Darmstadt, Germany). The plate was developed using solvent system - ethyl acetate: methanol: water (77:15:8). The zones were detected by examination under UV light (λ= 254nm) and in daylight after spraying with 0.5% anisaldehyde in methanol: glacial acetic acid: concentrated sulphuric acid (85:10:5) and subsequent heating at 100^0C for 10 minutes.

2.4 Results

2.4.1 Different varieties of unripe fruit of Momordica charantia L.

Since this research study commenced, a number of varieties of *Momordica charantia* (karela) fruit from different geographical origins were obtained. They were either home grown (UK) or imported from countries such as Thailand, Kenya, India, Nigeria and Bangladesh (Fig. 2.1-2.7). Surprisingly, karela fruit from different geographical sources were found to be different in size, shape and colour (Table 2.1). For example, karela fruit from India (Jaipur and Bombay), Africa (Kenya and

Table 2.1: Physical variation among different varieties of karela fruit

Varieties of karela fruit	*Colour of fruit*	*Dimensions of fruit:*		*Fruit pointed at:*	
		Length (cm)	*Max. width (cm)*	*Both ends*	*Apical end only*
Thai (Fig. 2.1)	Light green	26-33	6-7.5	No	Yes
Kenyan (Fig. 2.2)	Dark green	11-27	2.5-5.5	Yes	No
Indian (Jaipur) (Fig. 2.3)	Dark green	5-9	2-3.5	Yes	No
Indian (Bombay) (Fig. 2.4)	Dark green	7-12	2-4	Yes	No
Nigerian (Fig. 2.5)	Dark green	1-4	1-2.5	Yes	No
Bangladesh (Fig. 2.6)	Dark green	16-22	4-6	Yes	No
UK grown (Fig. 2.7)	Green	8-14	5-6	No	Yes

Nigeria) and Bangladesh had similar appearances; all were dark green in colour, pointed at both ends, covered in small warts with spiky irregular ridges running along their length. The major differences among them was the size. The Indian karela fruit (Fig. 2.3 and 2.4) were much smaller than the ones from Kenya (Fig. 2.2) and Bangladesh (Fig. 2.6), while the Nigerian karela fruit (Fig. 2.5) were the smallest among all different varieties. In contrast, Thai karela fruit (Fig. 2.1) were large in size, light green in colour, pointed only at the apical end, with smooth irregular ridges running along its surface. The home grown (UK) karela fruit (Fig. 2.7) were similar to those from Thailand, though they were green in colour and smaller in size. Despite the differences in appearance, their identities as *Momordica charantia* L. were confirmed by Dr Charles Jeffrey, a Cucurbitaceae expert at Royal Botanic Gardens, Kew, UK. A possible explanation for the physical differences among all these varieties is that they were different cultivars of *Momordica charantia*, i.e. they were grown in different parts of the world and the different weather conditions and agricultural techniques may account for the differences observed. Dr Jeffrey also commented that the Thai, Kenyan, Bangladesh and British varieties were likely to be cultivated, while the Indian and Nigerian varieties (being small in size) appeared to have grown in the wild. Furthermore, the author later came across (from a Chinese magazine) a new variety of karela fruit cultivated in the greenhouse in Taiwan. The fruit were very similar in shape to those from Kenya, however they were white in colour (Fig. 2.9).

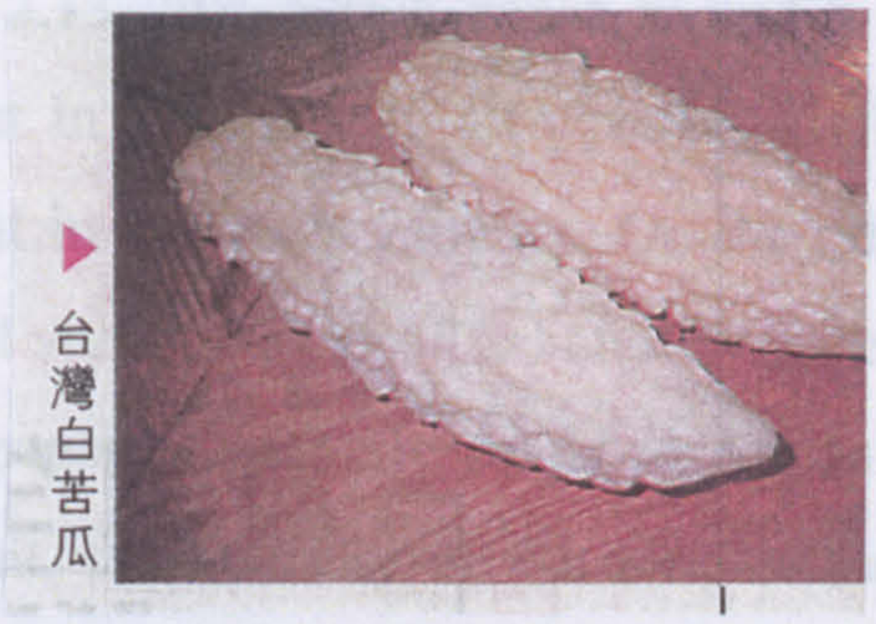


Figure 2.9: White karela fruit cultivated in Taiwan
(Photo reproduced from a Chinese magazine)

Dr Jeffrey emphasised that when classifying plants, the whole plant (leaves, flowers, fruit, seeds, stems, etc.) should be taken into account (Jeffrey, 1967). To investigate

this point, seeds obtained from three varieties of karela fruit (Thai, Indian (Jaipur) and Kenyan) were planted in a greenhouse in Chelsea Physic Garden, UK. The plants reached a height of about 7-8 feets after 5 months of growth, bearing flowers but no fruit. All three varieties of *Momordica charantia* plants were almost identical in appearance: climbing vines with bright green lobed leaves, long spirally twisted tendrils and small yellow flowers (Fig. 2.10-2.12). This again confirmed their identities as *M. charantia* L. despite the differences in physical appearances among the karela fruit.

2.4.2 Comparison of chemical constituents among different varieties of karela fruit

A simple assessment of the variation in chemical constituents among different varieties of karela fruit (Bangladesh, Kenyan, Thai, UK grown and Indian (Bombay)) was made using thin layer chromatography. From the results shown in Fig. 2.13, both qualitative and quantitative differences were observed. The pattern of the zone profiles were generally similar among the five varieties, however, some notable variations were seen. For example, the UV light absorbing zone **a** was present in all varieties except Indian karela, whereas the UV absorbing zones **b, c** and **d** were only present in UK, Indian and Bangladesh karela respectively. The UV absorbing zones **e** and **f** were present in all varieties except UK karela. Anisaldehyde is a spray reagent for detection of sugars, steroids and terpenes (Krebs *et al*, 1969). From the zone profiles shown in Fig. 2.13, two purple zones **w** and **x** (likely to be steroids or terpenoids) were present in Thai karela, also present (only in small quantities) in Indian karela, but absent in the other varieties. On the other hand, a bright yellow zone **y** was present in all varieties except Thai and UK karela, whereas a pink zone **z** (may be steroid or terpenoid) was only found in Indian karela. Where common constituents were present, the intensity of the zone differed among varieties, e.g. zones **s** and **t**. These results suggest that significant variations in chemical constituents occurred in karela fruit from different geographical origins.

2.5 Discussion and conclusion

Although its native country is uncertain, *Momordica charantia* is commonly

Figure 2.10: *Momordica charantia* plant of Kenyan origin

Figure 2.11: *Momordica charantia* plant of Indian origin

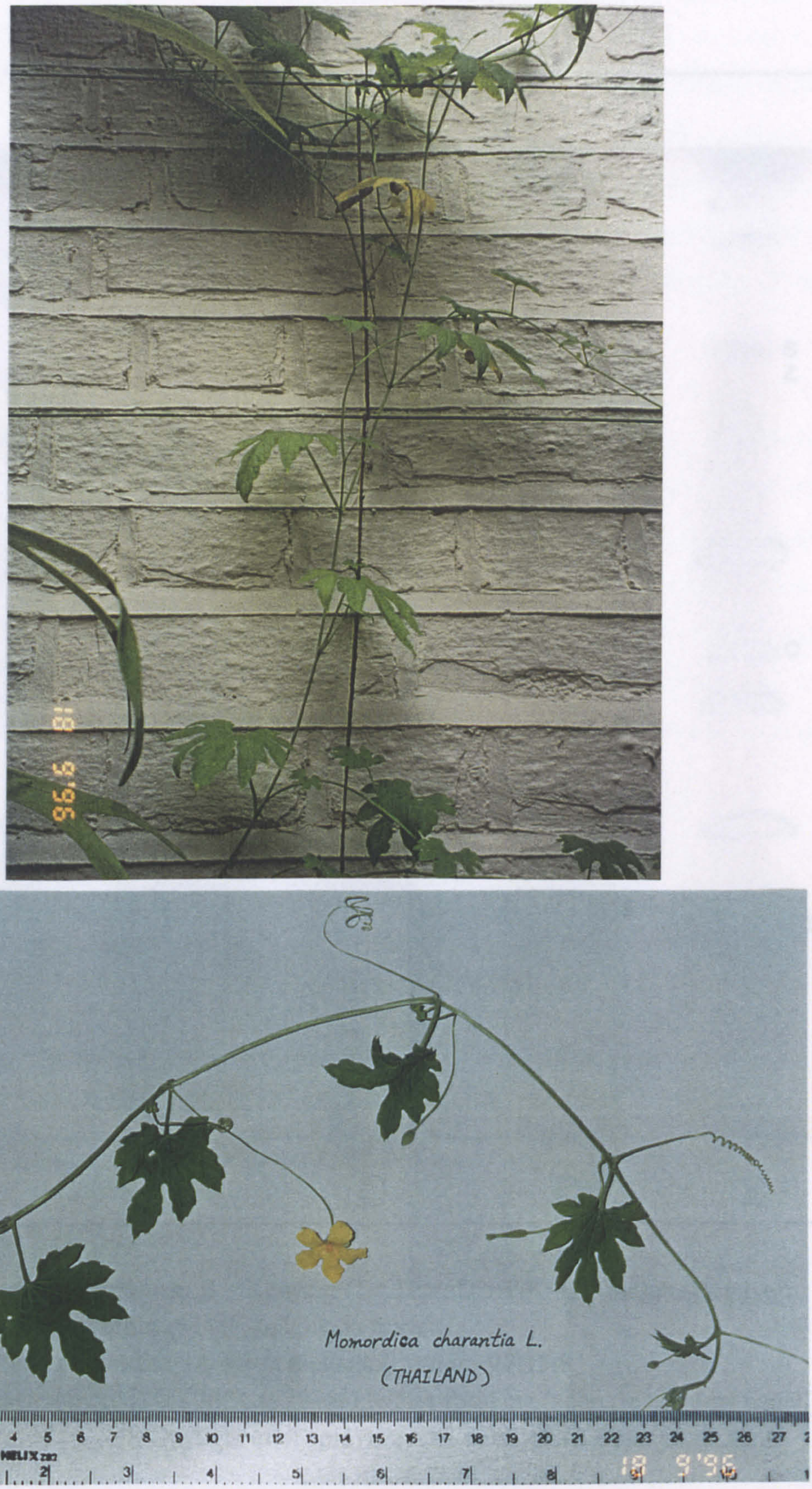
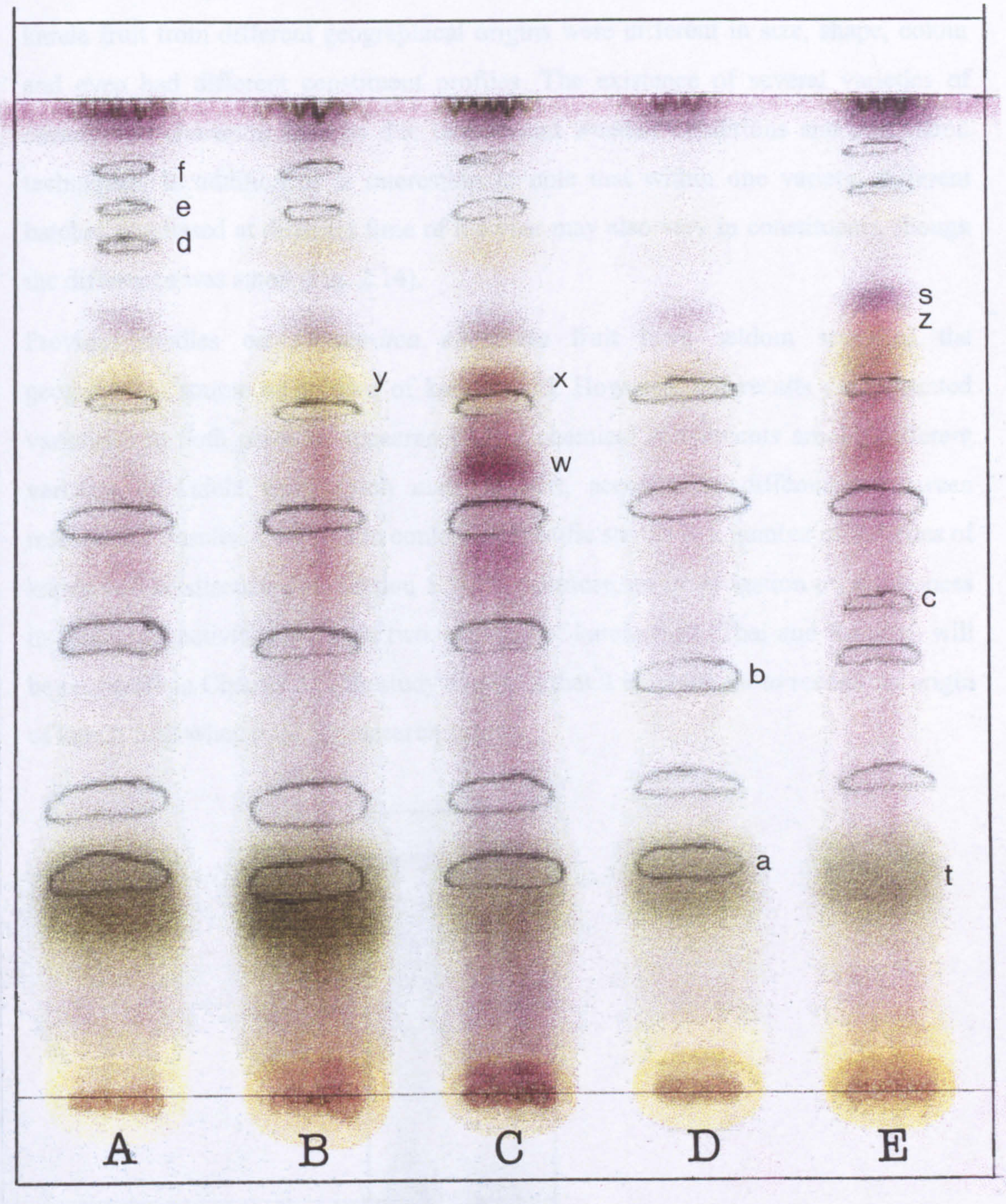


Figure 2.12: *Momordica charantia* plant of Thai origin



Keys: A = Bangladesh; B = Kenyan; C = Thai; D = UK; E = Indian (Bombay)
Adsorbent: Silica gel GF_{254}, 250μm thick
Mobile phase: Ethyl acetate: methanol: water - 77:15:8
Visualisation: i) UV light at 254nm before spraying (zones marked by pencil)
ii) Daylight after spraying with anisaldehyde spraying agent

Figure 2.13: The zone profiles of the juice from 5 different varieties of karela fruit.

cultivated throughout the tropics for the unripe fruit as food crop. We observed that karela fruit from different geographical origins were different in size, shape, colour and even had different constituent profiles. The existence of several varieties of *Momordica charantia* may be due to different weather conditions and cultivation techniques. In addition, it is interesting to note that within one variety, different batches purchased at different time of the year may also vary in constituents, though the difference was small (Fig. 2.14).

Previous studies on *Momordica charantia* fruit have seldom specified the geographical source or cultivar of karela used. However, our results demonstrated variations in both physical appearances and chemical constituents among different varieties of karela fruit which may, in part, account for differences between researchers' results. Variation in content of specific sterols in a number of varieties of karela will be discussed in Section 5.7. Furthermore, an investigation on differences in biological activities between two varieties of karela fruit (Thai and Kenyan) will be presented in Chapter 3. This study confirms that it is important to record the origin of karela fruit when used for research studies.

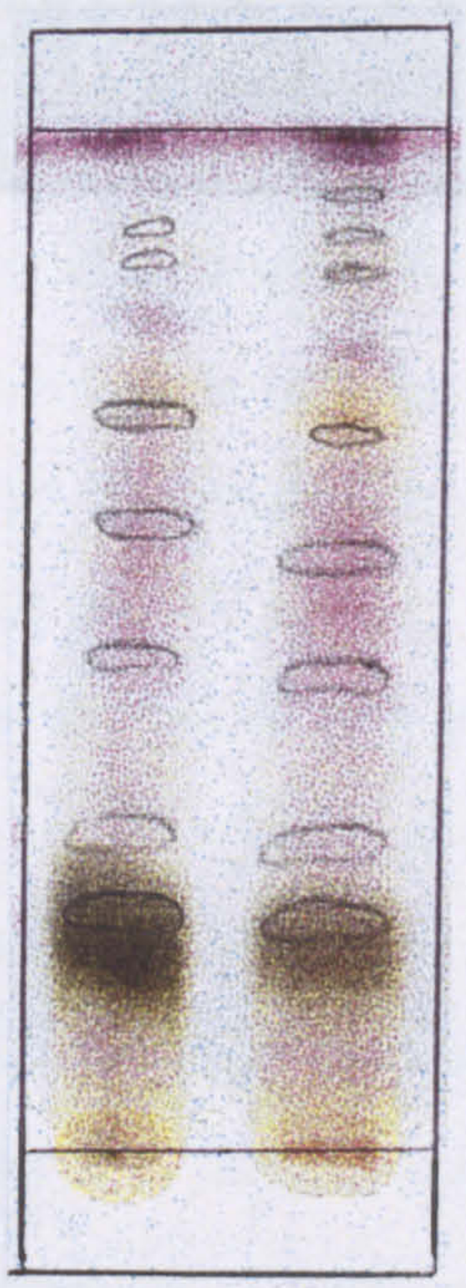


Figure 2.14: The zone profiles of fruit juice of two different batches of Kenyan karela. (Same TLC system used as in Fig. 2.13)

Chapter 3:

<u>In vivo</u> studies on karela fruit

Chapter 3: In vivo studies on karela fruit

3.1 Introduction

The main aim of the present study was to investigate the potential efficacy of karela fruit and its components in the treatment of Type II diabetes; the hypoglycaemic and anti-hyperglycaemic activities of karela. The ideal way of doing this would be using large scale double-blinded clinical trials. However, this is infeasible in the present work; the reasons have been discussed earlier on in Chapter 1. Thus the use of animal models of non-insulin-dependent diabetes would be an alternative for examining the effects of karela fruit on NIDDM.

3.1.1 Animal models of non-insulin-dependent diabetes

There are various animal models of non-insulin-dependent diabetes available (Bailey and Flatt, 1990; Shafrir, 1992). Selection of models for studying anti-diabetic compounds depends upon the particular features of NIDDM required.

According to Bailey and Flatt (1990), the models can be categorised into 3 main types:

a) Experimentally induced NIDDM models – they are produced by surgical (< about 95% pancreatectomy) or chemical (low-dose alloxan or streptozotocin, or neonatal streptozotocin) reduction of the β-cell mass.

b) Spontaneous syndromes of severe hyperglycaemia with possible ketosis – for example, Chinese hamster, the C57BL/KsJ db/db mouse and the Keeshond dog. These models usually show an early phase of obesity and hyperinsulinaemia with β-cell hyperplasia and insulin resistance. The hyperglycaemia usually exceeds 20 mmol/L.

c) Spontaneous syndromes of non-severe hyperglycaemia without ketosis – examples such as Zucker fatty fa/fa rat, Japanese KK mouse and Goto-Kakizaki rat. The first 2 of these models generally exhibit obesity, islet β-cell hyperplasia, hyperphagia, hyperinsulinaemia and insulin resistance. The hyperglycaemia is usually below 20 mmol/L.

3.1.2 The n0 STZ diabetic rat model

In the present study, the non-insulin-dependent diabetic animal model employed for examining the anti-diabetic activity of karela fruit is known as n0 STZ, i.e. an animal model of NIDDM induced by neonatal administration of streptozotocin (STZ). Streptozotocin, a 2-deoxymethyl-nitrosourea-glucopyranose molecule (Fig. 3.1), produces a selective toxic effect on pancreatic β-cells and induces diabetes mellitus in laboratory animals (Fischer, 1985). STZ is believed to act on nuclear DNA. During decomposition of STZ, highly reactive carbonium ions are formed which cause alkylation of DNA bases. In the following phase of excision DNA repair, the nuclear enzyme poly (ADP-ribose) synthetase becomes activated to such an extent that cellular levels of its substrates NAD become critically depleted, resulting in cell death (Le Doux *et al.*, 1986).

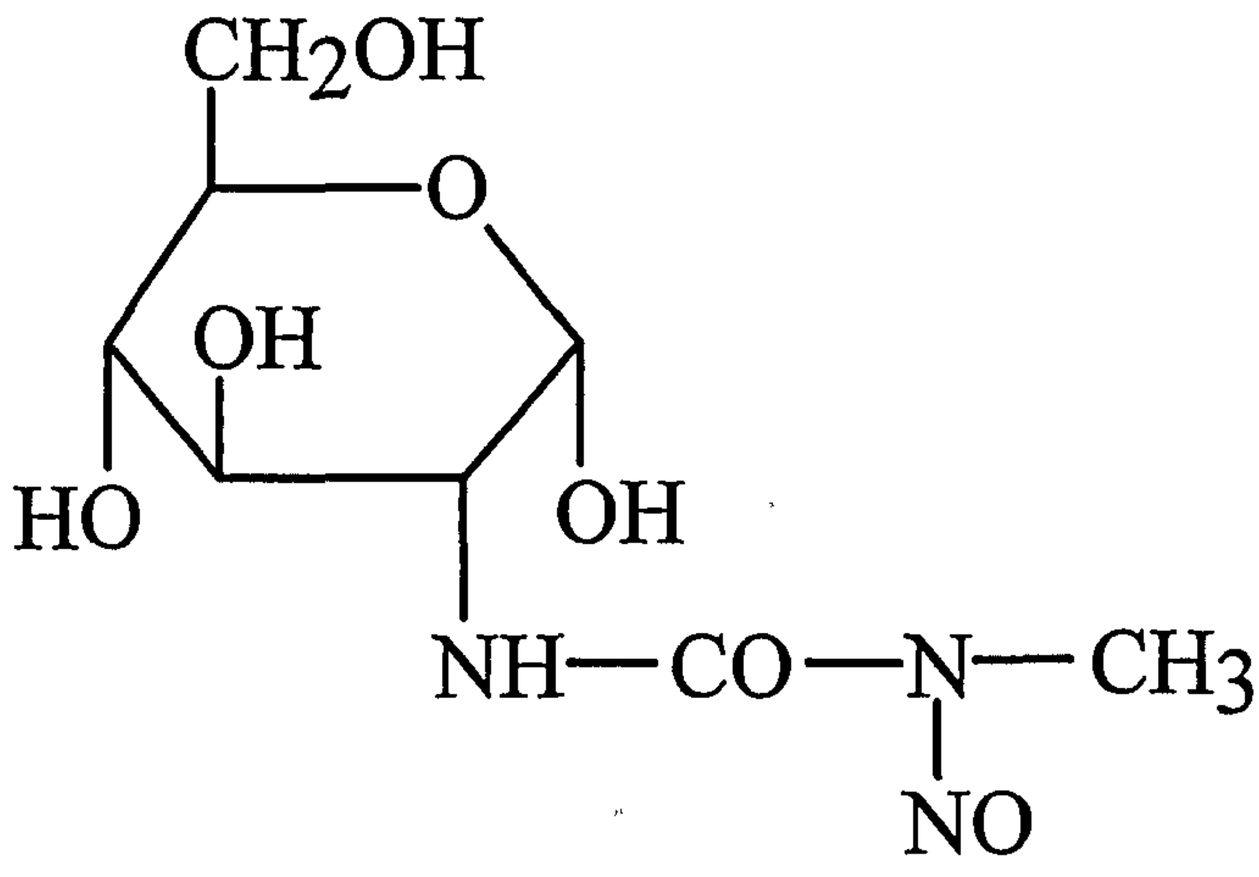


Figure 3.1: Structure of streptozotocin

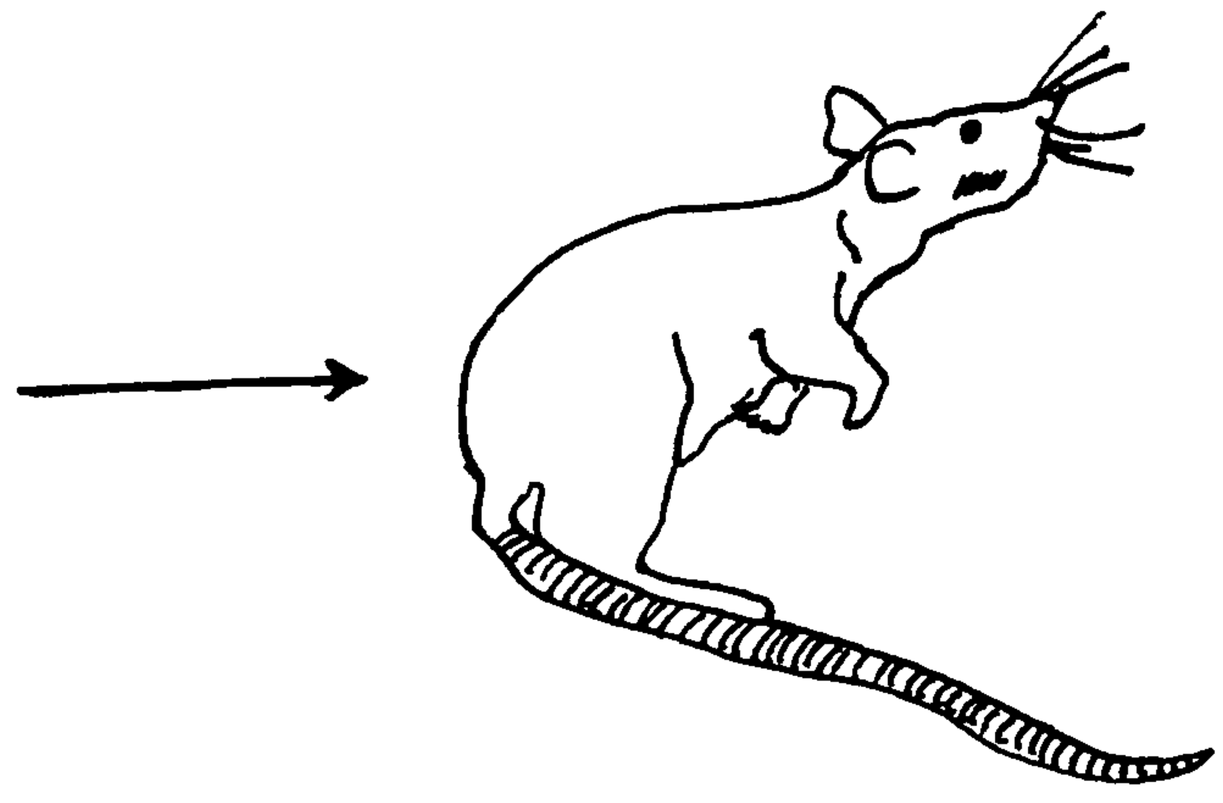
The diabetic syndrome in the n0 STZ model was generated by injecting Wistar rats on the day of birth (n0 = birth) intravenously (saphenous vein) with 100mg/kg body weight of streptozotocin (Portha *et al.*, 1979). This model is based on the fact that a capacity of regeneration of beta cells is still present in Wistar rats in the first week of life. In n0 STZ model, 50% of β-cell mass regenerates, and thus results in rats which are mildly hyperglycaemic with many characteristic features close to human NIDDM (Portha *et al.*, 1989; Fig. 3.2). This pattern of NIDDM is chronic and stable (up to 20 weeks).

This particular n0 STZ diabetic rat model was chosen in our study because this is a

Birth (day 0)

50% beta cell mass regenerate

Adult



<u>Stable diabetic state:</u>

- mild hyperglycaemia
- mild hypoinsulinaemia
- glucose intolerance
- no insulin resistance
- specific failure of insulin release in response to glucose

Figure 3.2: The n0 STZ NIDDM model

standard diabetic model used at Lipha (the industrial sponsor of this research project) for screening anti-diabetic compounds. The advantage of this model is that the regeneration of 50% β-cell mass results in a mildly hyperglycaemic model, with specific failure of insulin release in response to glucose. In addition, the running costs for experiments which involve rats are relatively cheap as compared to other animal species such as rabbits or gerbils. However, the limitation of this model is that the rats do not have the complete characteristic features of NIDDM, for instance, the rats have no insulin resistance.

3.2 Effect of oral karela juice on basal glycaemia and oral glucose tolerance

3.2.1 Materials and methods

Plant material

Two different varieties of fresh unripe karela fruit, imported from Thailand (Fig. 2.1) and Kenya (Fig. 2.2), were purchased from local grocery stores (Section 2.2). Both varieties were authenticated by Dr Charles Jeffrey (Cucurbitaceae expert, Royal Botanic Gardens, Kew, UK). Herbarium voucher specimens (no. Mo10L1 & Mo10L2) were deposited at the Pharmacognosy Section, Department of Pharmacy, King's College London.

Preparation of karela juice

Fresh fruit were thoroughly washed and seeds removed. The fruit flesh was cut into small pieces, crushed in a kitchen blender, and the juice collected after filtering through muslin cloth. Karela juice was kept refrigerated (2-8 ^{O}C) and used within 5 days. For 200ml juice, approximately 0.4kg fresh fruit of Thai karela or 0.9kg fresh fruit of Kenyan karela (without seeds) was required.

Animals

Adult Wistar non-insulin-dependent diabetic (NIDD) rats, 3-4 months old, weighing 245-335g and bred at the animal house at Lipha were used. They were maintained on standard rat pellets (UAR, Villemoisson sur Orge, France; diet no. UAR 113) and tap water ad libitum. The rats were made diabetic by injecting streptozotocin (STZ;

Sigma, see Appendix A) intravenously (100mg/kg body weight) on the day of birth (n0 STZ; Section 3.1.2). The induction of STZ-diabetes was confirmed by the determination of fasting hyperglycaemia (≥ 25% above normal value which is around 140mg/dl[1]) on Control Day.

Effects on basal glycaemia

On Control Day, water (10ml/kg) was orally administered (force-feed with a syringe) to 1h fasted n0 STZ rats at 0 min, and blood samples (0.3ml) were drawn from the tail tip at 60, 90, 120, 180, 240 and 360 minutes. The rats were kept unfed throughout this period.

Rats with plasma glucose ≥ 140mg/dl[1] at 1 hour sampling time were chosen for the test, and randomly divided into various groups.

On Test Day 1 (4 days after Control Day), rats (n=5) were orally dosed with one of the following: water (10ml/kg body weight) as a negative control; metformin (200mg/kg; Lipha), a well-known anti-diabetic agent (Section 1.1.9(b)), as a positive control; Thai karela juice (5ml or 10ml/kg) or Kenyan karela juice (5ml or 10ml/kg). Blood sampling was carried out at the same time points as on Control Day.

Apart from studying the acute effect of karela juice by single dose administration on Test Day 1, the cumulative effect of karela juice was also examined. In order to investigate the effect of cumulative administration of karela juice, the different treatments were given for four consecutive days and the experimental procedures were repeated on Test Day 4.

Effects on oral glucose tolerance test (OGTT)

On Control Day, water (10ml/kg) was orally administered to 2h fasted n0 STZ rats, 30 min before an oral glucose challenge (2g/kg body weight; 2g in 5ml water; Merck). Blood samples (0.3ml) were drawn from the tail tip at 0 min (just before glucose was given) and at 10, 20, 30, 45, 60 and 90 min after glucose administration. The rats were kept unfed throughout this period.

Rats with high ΔG values (≥ 400mg/dl; ΔG = the sum of the increase in plasma

[1] Units for glucose measurements: mg/dl = mmol/L × 18

glucose at all time points compared with time 0) were chosen for the experiment, and randomly divided into various groups.

On Test Day 1 (3 days after Control Day), rats (n=6) were orally dosed (force-feed with a syringe) with one of the following: water (10ml/kg body weight) as a negative control, metformin (200mg/kg; Lipha) as a positive control, Thai karela juice (10ml/kg) or Kenyan karela juice (5ml/kg)*. As on Control Day, oral glucose was given 30 min after the test substances and blood sampling was carried out at the same time points.

To investigate the effect of cumulative administration of karela juice, the four different treatments were given for four consecutive days and the experimental procedures for OGTT repeated on Test Day 4.

* From the preparation of karela juice, it was found that in order to obtain the same volume of juice, the ratio of weight of fruit flesh of Kenyan to Thai karela was approximately 2:1. Thus the dosage of karela juice used in our testing was 5ml/kg (Kenyan) and 10ml/kg (Thai), so that a similar original weight of karela of the two different varieties were tested.

Analysis of plasma glucose and insulin

Blood samples were subjected to centrifugation (9000rpm; 5 min) and plasma separated from blood cells. Plasma glucose was measured with a glucose analyser (EBIO Analyser 6666 or Beckman Glucose Analyser 2) using a glucose oxidase method. Plasma insulin was measured by radioimmunoassay using a commercial kit (Sorin-Insik 5) with rat insulin (Novo Nordisk) as standard.

In both studies (effects on basal glycaemia and oral glucose tolerance), results obtained for each treatment group on Control Day were compared with the results of that group on Test Days 1 or 4. Data obtained were evaluated using Student's paired t-tests so that each individual animal's results on Test and Control days were compared. Values were considered to be significantly different if $p < 0.05$.

3.2.2 Results

3.2.2 (a) Effects of karela juice on basal glycaemia

The effects of water and metformin on basal glycaemia in n0 STZ diabetic rats are

shown in Table 3.1. Water (negative control) did not significantly decrease the mean plasma glucose at any time point on either Test Days 1 or 4. On the other hand, with metformin (an anti-diabetic drug which acts as a positive control in this study), there was a significant decrease at most time points on both Test Days 1 and 4. These results validate our experimental model.

From the results (Table 3.2), for Thai karela juice 5ml/kg, there was no significant decrease in mean plasma glucose on Day 1. On Day 4, there was a decrease in mean plasma glucose at 60, 90 and 120 min but none of these decreases were statistically significant ($p > 0.05$). When the dosage was increased to 10ml/kg, very similar results were obtained on Day 1. However, on Day 4, there was a larger decrease in mean plasma glucose at 60, 90 and 120 min ($p = 0.15$, 0.10 and 0.25 respectively) but none of them reached statistical significance at the accepted level of $p < 0.05$. These results indicated that Thai karela may have a cumulative effect on basal blood glucose and a higher dose may result in a statistically significant effect. It is also interesting to note that at both dosages, there was an unexpected significant increase in mean plasma glucose at 360 min on Day 1, for which there was no clear explanation.

In the case of Kenyan karela juice (Table 3.3), at doses of both 5 and 10ml/kg, there was no significant difference in mean plasma glucose at any time points on either Test Days 1 or 4.

From the negative results of both varieties of karela on basal glycaemia, it is not surprising to find that both varieties (at both dosages) had no statistically significant ($p > 0.05$) effect on the basal insulin levels (Tables 3.4 and 3.5). However, the fact that there was an increase (though not statistical significant; $0.05 < p < 0.1$) in mean plasma insulin level for Thai karela juice at dose of 5ml/kg (Test Day 1) and Kenyan karela juice at dose of 5ml/kg (both Days 1 and 4) at some time points suggests that insulin may have been released.

3.2.2 (b) Effects of karela juice on oral glucose tolerance

The effects of water and metformin on plasma glucose in the oral glucose tolerance study in n0 STZ diabetic rats are shown in Table 3.6. Water (negative control) did

Table 3.1: Effect of orally administered water (10ml/kg body weight) and metformin (200mg/kg body weight; 200mg in 10ml water) on basal glycaemia in 1h fasted n0 STZ diabetic rats.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM) at time from administration of test substance:					
	60 min	90 min	120 min	180 min	240 min	360 min
Water						
Control Day (n=5)	155.6 ± 6.3	170.6 ± 5.5	173.6 ± 8.9	150.6 ± 3.7	147.8 ± 3.8	136.0 ± 9.1
Day 1 (n=5)	174.0 ± 4.2**	192.4 ± 9.5*	188.0 ± 11.0	142.2 ± 5.9	141.4 ± 5.5	137.4 ± 6.1
Day 4 (n=5)	157.6 ± 3.8	160.6 ± 3.5	164.8 ± 9.7	144.6 ± 3.1	143.6 ± 5.9	136.4 ± 5.4
Metformin						
Control Day (n=5)	156.6 ± 6.1	179.4 ± 7.1	192.4 ± 8.5	161.0 ± 10.8	151.6 ± 6.9	143.4 ± 2.9
Day 1 (n=5)	130.8 ± 2.5***	143.8 ± 5.2***	147.6 ± 7.6*	128.8 ± 5.0*	125.4 ± 1.8*	128.6 ± 2.6*
Day 4 (n=4)	131.8 ± 1.3*	140.3 ± 3.9***	132.3 ± 5.9*	127.0 ± 2.9	128.0 ± 3.3*	130.8 ± 3.8

Table 3.2: Effect of orally administered Thai karela juice (5ml or 10ml/kg body weight) on basal glycaemia in 1h fasted n0 STZ diabetic rats.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM) at time from administration of test substance:					
	60 min	90 min	120 min	180 min	240 min	360 min
Dose: 5ml/kg						
Control Day (n=5)	163.0 ± 9.0	180.8 ± 15.5	187.4 ± 20.4	146.8 ± 6.2	137.6 ± 5.7	124.0 ± 3.3
Day 1 (n=5)	163.2 ± 7.2	179.8 ± 12.1	199.0 ± 11.8	155.6 ± 6.8	148.4 ± 6.7	141.0 ± 4.0*
Day 4 (n=5)	158.0 ± 3.9	174.0 ± 11.4	172.2 ± 7.1	146.4 ± 5.8	143.6 ± 5.4	133.0 ± 2.5
Dose: 10ml/kg						
Control Day (n=5)	159.6 ± 7.2	169.8 ± 3.9	188.0 ± 17.1	146.0 ± 4.3	134.2 ± 5.0	123.0 ± 5.4
Day 1 (n=5)	160.4 ± 7.1	172.0 ± 9.8	179.6 ± 4.9	160.8 ± 4.5	147.2 ± 7.2	133.8 ± 3.5**
Day 4 (n=5)	150.6 ± 6.3	159.0 ± 5.5	161.8 ± 3.8	149.2 ± 9.4	137.0 ± 8.4	122.8 ± 8.2

Table 3.3: Effect of orally administered Kenyan karela juice (5ml or 10ml/kg body weight) on basal glycaemia in 1h fasted n0 STZ diabetic rats.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM) at time from administration of test substance:					
	60 min	90 min	120 min	180 min	240 min	360 min
Dose: 5ml/kg						
Control Day (n=5)	157.2 ± 8.2	170.0 ± 8.6	175.4 ± 8.5	142.4 ± 6.9	138.0 ± 4.3	127.8 ± 6.8
Day 1 (n=5)	152.0 ± 3.7	167.2 ± 8.4	176.4 ± 9.6	152.6 ± 6.8	146.6 ± 5.6	138.8 ± 6.1
Day 4 (n=4)	158.5 ± 6.5	163.0 ± 7.9	170.0 ± 8.9	153.3 ± 0.8	146.5 ± 6.2	143.3 ± 7.1
Dose: 10ml/kg						
Control Day (n=5)	161.6 ± 7.5	179.0 ± 9.2	208.5 ± 15.4	158.4 ± 7.8	157.2 ± 5.9	145.0 ± 5.9
Day 1 (n=5)	174.4 ± 15.8	186.2 ± 18.8	195.0 ± 27.0	163.4 ± 16.4	155.0 ± 8.4	142.4 ± 6.9
Day 4 (n=5)	157.8 ± 9.2	172.6 ± 8.8	189.5 ± 19.3	155.8 ± 10.5	155.4 ± 7.6	144.2 ± 4.8

Table 3.4: Effect of orally administered Thai karela juice (5ml or 10ml/kg body weight) on basal insulinaemia in 1h fasted n0 STZ diabetic rats.

*p < 0.1, **p < 0.05, ***p < 0.02, ****p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM) at time from administration of test substance:					
	60 min	90 min	120 min	180 min	240 min	360 min
Dose: 5ml/kg						
Control Day (n=5)	233.4 ± 18.8	281.0 ± 32.0	225.8 ± 21.0	280.0 ± 27.1	253.4 ± 26.3	246.0 ± 14.8
Day 1 (n=5)	291.4 ± 33.1	344.2 ± 25.5	290.4 ± 19.0*	351.8 ± 15.2*	322.4 ± 17.1*	290.4 ± 12.7*
Day 4 (n=5)	292.8 ± 26.1*	316.0 ± 28.1	291.2 ± 25.0	283.0 ± 25.1	274.4 ± 26.4	287.6 ± 9.4
Dose: 10ml/kg						
Control Day (n=5)	220.6 ± 38.6	250.5 ± 70.6	203.4 ± 36.3	228.4 ± 37.5	214.2 ± 38.9	189.4 ± 32.7
Day 1 (n=5)	272.6 ± 61.3	231.8 ± 40.8	238.4 ± 38.2	252.6 ± 42.5	246.0 ± 36.6	237.4 ± 28.5*
Day 4 (n=5)	239.0 ± 42.4	258.2 ± 43.4	224.2 ± 39.3	293.8 ± 78.7	206.2 ± 41.0	177.2 ± 36.3

Table 3.5: Effect of orally administered Kenyan karela juice (5ml or 10ml/kg body weight) on basal insulinaemia in 1h fasted n0 STZ diabetic rats.

*p < 0.1, **p < 0.05, ***p < 0.02, ****p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM) at time from administration of test substance:					
	60 min	90 min	120 min	180 min	240 min	360 min
Dose: 5ml/kg						
Control Day (n=5)	261.6 ± 14.1	271.0 ± 10.4	209.8 ± 17.3	242.6 ± 26.9	241.2 ± 28.1	241.0 ± 21.4
Day 1 (n=5)	293.0 ± 4.8*	295.4 ± 12.4	269.2 ± 26.8*	286.2 ± 20.6	282.2 ± 21.6	269.4 ± 15.9
Day 4 (n=5)	298.0 ± 21.0*	316.6 ± 16.4	281.6 ± 33.0*	307.0 ± 26.8	289.2 ± 15.0	281.4 ± 28.8
Dose: 10ml/kg						
Control Day (n=5)	291.4 ± 48.9	316.0 ± 79.2	266.0 ± 43.3	301.2 ± 47.6	300.8 ± 57.3	272.4 ± 38.0
Day 1 (n=5)	288.8 ± 58.7	310.0 ± 49.8	281.8 ± 49.8	311.8 ± 65.6	272.8 ± 32.7	255.4 ± 37.7
Day 4 (n=5)	225.0 ± 34.4	226.2 ± 33.7	288.8 ± 55.1	295.8 ± 49.9	248.8 ± 35.2	252.8 ± 26.3

Table 3.6: Effect of orally administered water (10ml/kg body weight) and metformin (200mg/kg body weight; 200mg in 10ml water) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; water or metformin was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Water							
Control Day (n=6)	147.0 ± 5.8	236.8 ± 7.5	272.8 ± 18.4	283.5 ± 19.2	283.5 ± 26.3	256.5 ± 23.3	201.7 ± 15.6
Day 1 (n=6)	162.7 ± 9.9	230.7 ± 8.3	255.0 ± 12.5	267.7 ± 13.2	251.5 ± 17.0	246.8 ± 17.6	214.0 ± 16.3
Day 4 (n=6)	140.0 ± 3.2	244.0 ± 7.9	265.2 ± 12.1	274.3 ± 21.2	250.3 ± 22.1	237.7 ± 25.1	209.5 ± 19.4
Metformin							
Control Day (n=5)	155.0 ± 6.7	249.8 ± 7.8	272.2 ± 11.0	284.0 ± 19.6	282.8 ± 22.0	254.4 ± 20.4	196.2 ± 9.1
Day 1 (n=5)	160.0 ± 9.9	199.2 ± 9.2***	207.6 ± 11.6***	214.2 ± 15.2***	210.6 ± 15.1***	237.8 ± 37.4	165.8 ± 9.7***
Day 4 (n=4)	144.0 ± 5.2	199.8 ± 7.3***	206.0 ± 8.4**	214.3 ± 18.9	199.3 ± 13.8***	193.3 ± 7.7*	172.5 ± 3.6

not significantly affect glucose tolerance at any time point on either Days 1 or 4. On the other hand, with metformin, there was a significant decrease of mean plasma glucose at most time points on both Day 1 and Day 4. These results validate our experimental model.

Figure 3.3 shows the anti-hyperglycaemic effects of juice from both varieties of karela in n0 STZ diabetic rats. For Thai karela (Fig. 3.3a), the reduction in plasma glucose at individual time points on Test Day 1 did not reach statistical significance although mean values were lower than control. However, mean ΔG was significantly reduced by 29% ($p < 0.05$). Cumulative treatment resulted in a better improvement in oral glucose tolerance (Test Day 4); with a significant decrease in plasma glucose at 20, 30, 45 and 60 min and a reduction of mean ΔG by 38% ($p < 0.05$).

For Kenyan karela (Fig. 3.3b), single day treatment significantly decreased plasma glucose levels at 20, 30 and 45 min. Although mean plasma glucose values for the cumulative treatment were similar to the single treatment, these were not significantly different from control, probably due to greater variation in values. Neither acute nor cumulative administration of Kenyan karela produced a statistically significant reduction in mean ΔG.

The effects of both varieties of karela on plasma insulin levels are shown in Figure 3.4. For Thai karela (Fig. 3.4a), acute administration of the juice (Day 1) resulted in a large increase in mean plasma insulin at 10 min, though it did not reach statistical significance. However, cumulative administration caused a significant increase in phase I insulin secretion (0, 10 min).

For Kenyan karela (Fig. 3.4b), an increase in the overall area under the plasma insulin curve was observed on both Days 1 and 4 but differences from control did not reach statistical significance apart from at the 20 min time point on Day 4.

No significant effect on plasma insulin levels was observed with either acute or cumulative administration of water (Appendix 1) which validates our results.

3.2.3 Discussion

In most (if not all) previous animal studies on karela (Raman and Lau, 1996), experiments were designed so that separate control and treatment groups were used.

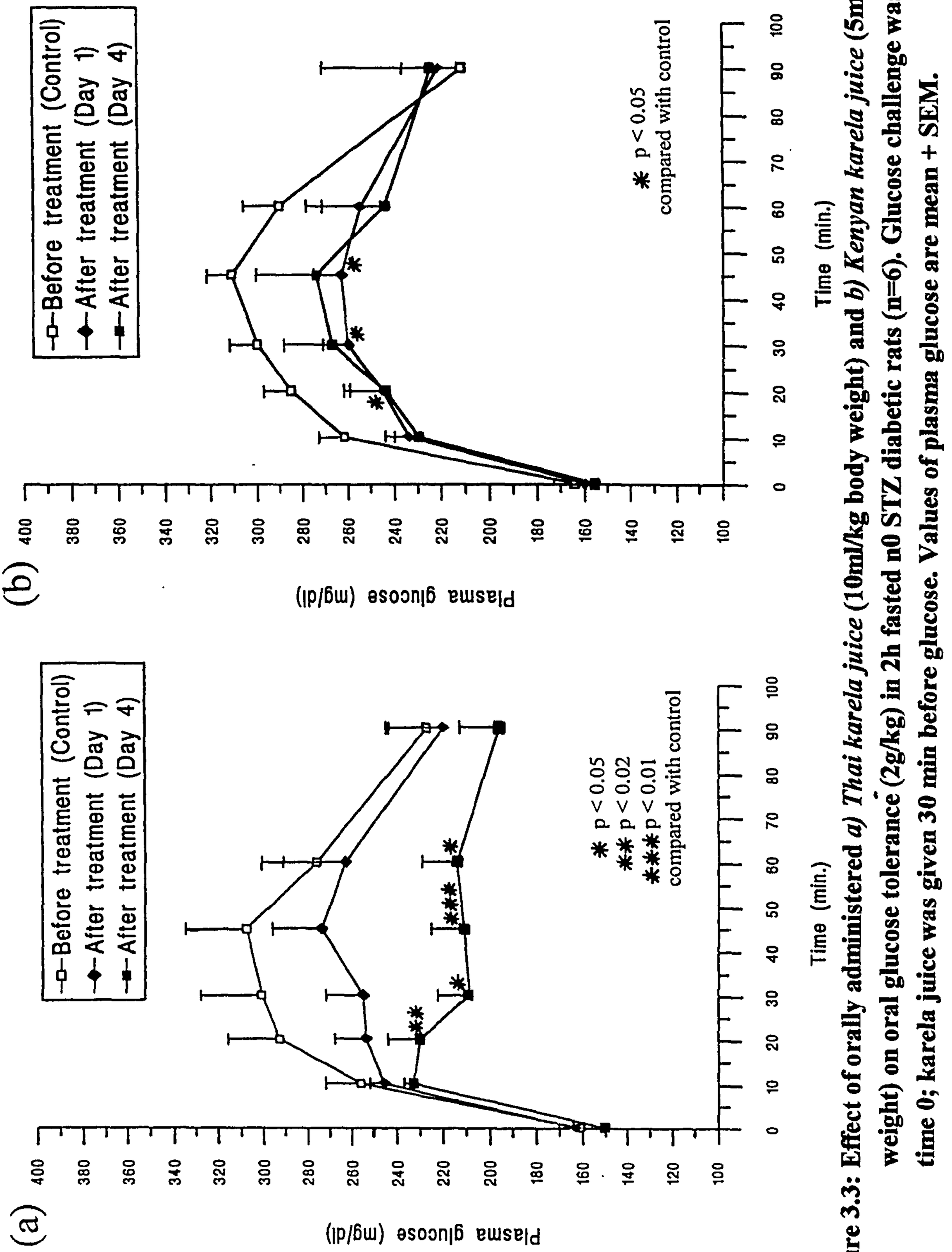
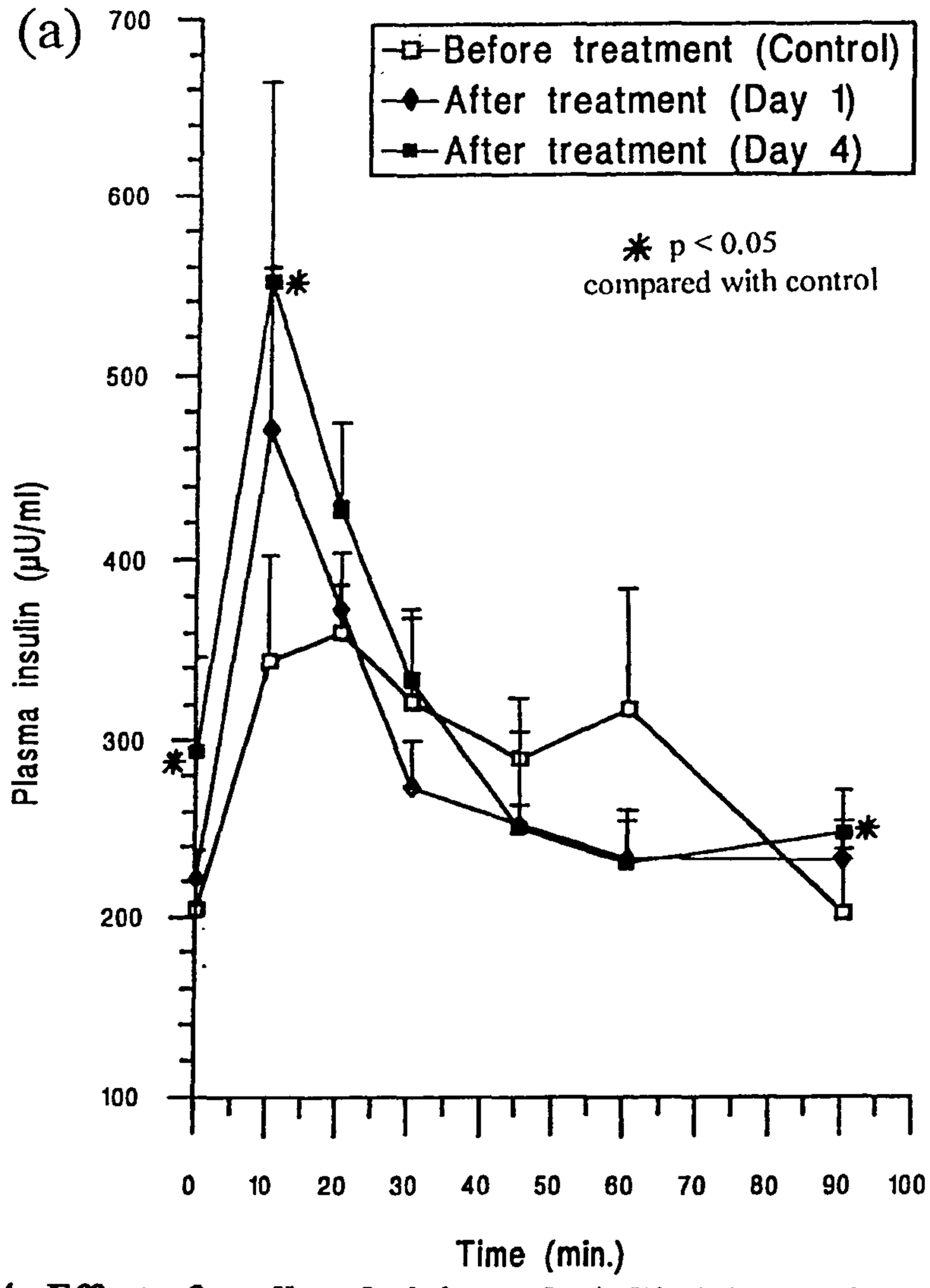


Figure 3.3: Effect of orally administered *a) Thai karela juice* (10ml/kg body weight) and *b) Kenyan karela juice* (5ml/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given 30 min before glucose. Values of plasma glucose are mean + SEM. time 0; karela juice was given 30 min before glucose challenge was given at



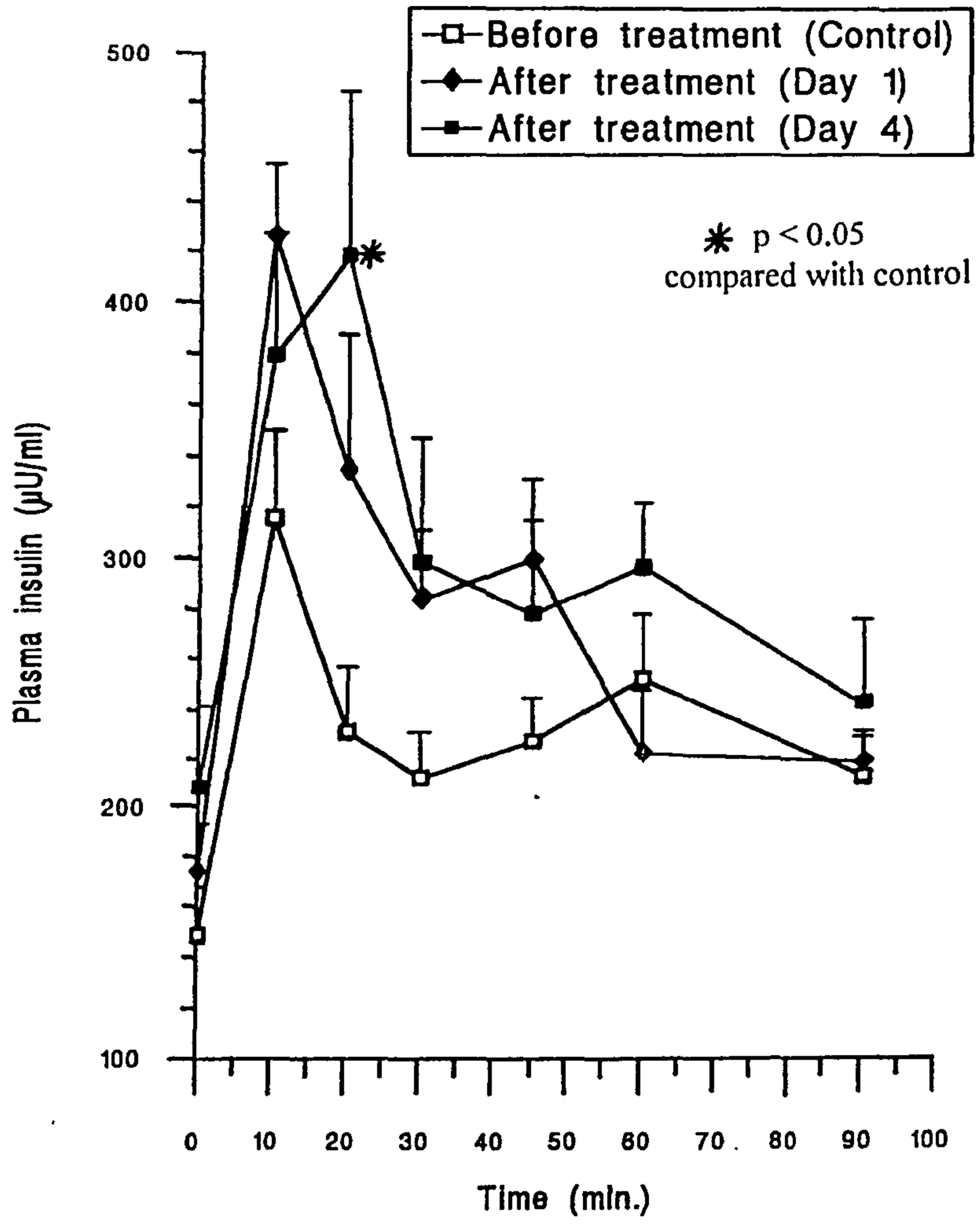


Figure 3.4: Effect of orally administered *a) Thai karela juice* (10ml/kg body weight) and *b) Kenyan karela juice* (5ml/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela juice was given 30 min before glucose. Values of plasma insulin are mean + SEM.

In contrast, in our present study, experimental design and the method of interpreting the results was to compare the results obtained within each treatment group on Control Day with Test Days 1 and 4. Since each rat may react differently despite the same experimental conditions, the advantage of this method was to compare the results of individual rats on Control Day with Test Days 1 and 4. Here, assumption was made that the same rat will react in the same way on different days provided the experimental conditions were kept constant. To demonstrate this, the mean plasma glucose of the 6 rats in which water was given on Control Day, Test Days 1 and 4 (in the oral glucose tolerance test) is given in Table 3.6. There was no significant difference either between the mean plasma glucose on Control Day and Test Day 1 or between the values on Control Day and Test Day 4. Furthermore, this method of interpretation only requires a small number of rats for each treatment group as there is no need to account for inter-individual variation.

From the results, both Thai and Kenyan karela did not produce a statistically significant effect on basal glycaemia on NIDDM model rats at the tested doses (5 or 10ml/kg body weight). In this context, our results were contradictory to some previous researchers who had demonstrated a hypoglycaemic effect on acute administration of karela juice/extracts in both normal animals (Leatherdale *et al.*, 1981; Karunanayake *et al.*, 1984; Chandrasekar *et al.*, 1989) and diabetic animals (Sharma *et al.*, 1960; Higashino *et al.*, 1992; Ali *et al.*, 1993a). However, our results indicated that Thai karela, but not Kenyan karela, showed an apparent cumulative decreasing effect on basal blood glucose and a statistically significant effect may be seen if higher dosages are used. Kulkarni and Gaitonde (1962) reported no reduction in fasting glucose levels on either acute or cumulative administration of dried karela juice to normal animals. These conflicting results may be due to variations in blood sampling times and the variety, dosages and preparations of karela used, as well as the diabetic status (IDDM, NIDDM or normal) of the animals.

In contrast, our results from the oral glucose tolerance test confirmed that both varieties exhibited anti-hyperglycaemic activities though not to the same extent. Both varieties of karela juice produced a significant improvement in OGTT, of which the effect of Thai karela was more pronounced. The results of plasma insulin

measurement showed that this improvement in OGTT was due, at least in part, to an increase in insulin secretion in response to glucose, mainly in the first phase.

In healthy (non-diabetic) humans, glucose administration is known to promote an initial (phase I) stimulation of insulin within the first 5-10 min (Section 1.1.7). In NIDDM patients however, there is an impairment in this first phase of insulin secretion (Cerasi *et al.*, 1972; Robertson, 1992). A similar deficiency is observed in the n0 STZ model used in the present study (Portha *et al.*, 1979; Giroix *et al.*, 1983), as shown in Fig. 3.4 (control graphs). In fact, these rats are mildly hyperglycaemic with many characteristics features close to human NIDDM (Fig. 3.2). Thus the improvement of both glucose tolerance and phase I insulin response in these animals may be of significance to the treatment of NIDDM patients using karela.

Based on an extensive literature search (Raman and Lau, 1996), it appears this is the first demonstration of an *in vivo* insulin secretagogue effect by karela despite reports of an effect *in vitro* (Welihinda *et al.*, 1982a,b; Ali *et al.*, 1993b; Mosihuzzaman *et al.*, 1994). The stimulation of phase I insulin secretion reported here, may have been missed in earlier studies on humans (Leatherdale *et al.*, 1981) or animals (Leatherdale *et al.*, 1981; Day *et al.*, 1990; Sarkar *et al.*, 1996), due to the later initial blood sampling times (30 min or more) employed previously. In addition, it is possible that stimulation of insulin secretion may not have been apparent in the particular models used. For instance, it may be masked in normal animals where a good phase I response already exists, or absent in models where almost complete destruction of β-cells has occurred (Day *et al.*, 1990; Sarkar *et al.*, 1996). The doses of STZ employed in these latter studies would be expected to have an insulinopenic effect (Bailey and Flatt, 1990). Further studies on insulin stimulation by karela will be discussed in the following section (Section 3.3). No statistically significant alterations to plasma insulin level were observed in studies on basal glycaemia (Section 3.2.2(a)) but this may be due to the late sampling time for the first blood sample (60 min after the administration of karela juice).

Since Thai karela (10ml/kg) gave a more significant improvement on oral glucose tolerance than Kenyan karela (5ml/kg), our further research studies (as discussed in the following chapters) were focused solely on Thai karela.

3.3 Investigation on the dose-response effect of karela on oral glucose tolerance

In the previous section (3.2.2(b)), both varieties (Thai and Kenyan) of karela were shown to improve oral glucose tolerance and stimulate phase I insulin secretion, though to a different extent. Thus, the dose-response effect of the two varieties of karela on oral glucose tolerance was examined.

3.3.1 Materials and methods

Freshly prepared karela juices(both varieties) were used in the study. The same n0 STZ NIDDM rat model was employed and an oral glucose tolerance test was carried out (same experimental procedures as described in Section 3.2.1). The dosages of karela juice (for both varieties) used were 5, 10 and 15ml/kg of body weight. As before, treatment was administered for 4 days and both acute (after single dose treatment) and cumulative (after 4 days of treatment) effects of karela on oral glucose tolerance were studied. Plasma glucose and insulin were measured. Water and metformin controls were performed as before (results not shown).

3.3.2 Results

Plasma glucose

From the results (Fig. 3.5; Table 3.7), for Thai karela juice, 5ml/kg dose improved oral glucose tolerance after single treatment but only reached statistical significance at 2 time points, with mean ΔG reduced by 28% ($p = 0.171$). However, cumulative treatment with 5ml dose improved oral glucose tolerance significantly at most time points, with mean ΔG reduced by 33% ($p = 0.065$) as compared with control. As the dose was increased to 10ml/kg, a similar result was obtained with the single dose where plasma glucose was only significantly reduced at 2 time points, with a mean ΔG reduction of 22% ($p = 0.153$). However the cumulative treatment had a more pronounced improvement in oral glucose tolerance, with a significant decrease in plasma glucose at all time points, where the mean ΔG was reduced by 17% ($p = 0.051$). As the dose was further increased to 15ml/kg, even a single dose produced a significant improvement in oral glucose tolerance at 4 time points, with a mean ΔG reduction of 27% ($p = 0.114$). The cumulative treatment reduced mean plasma

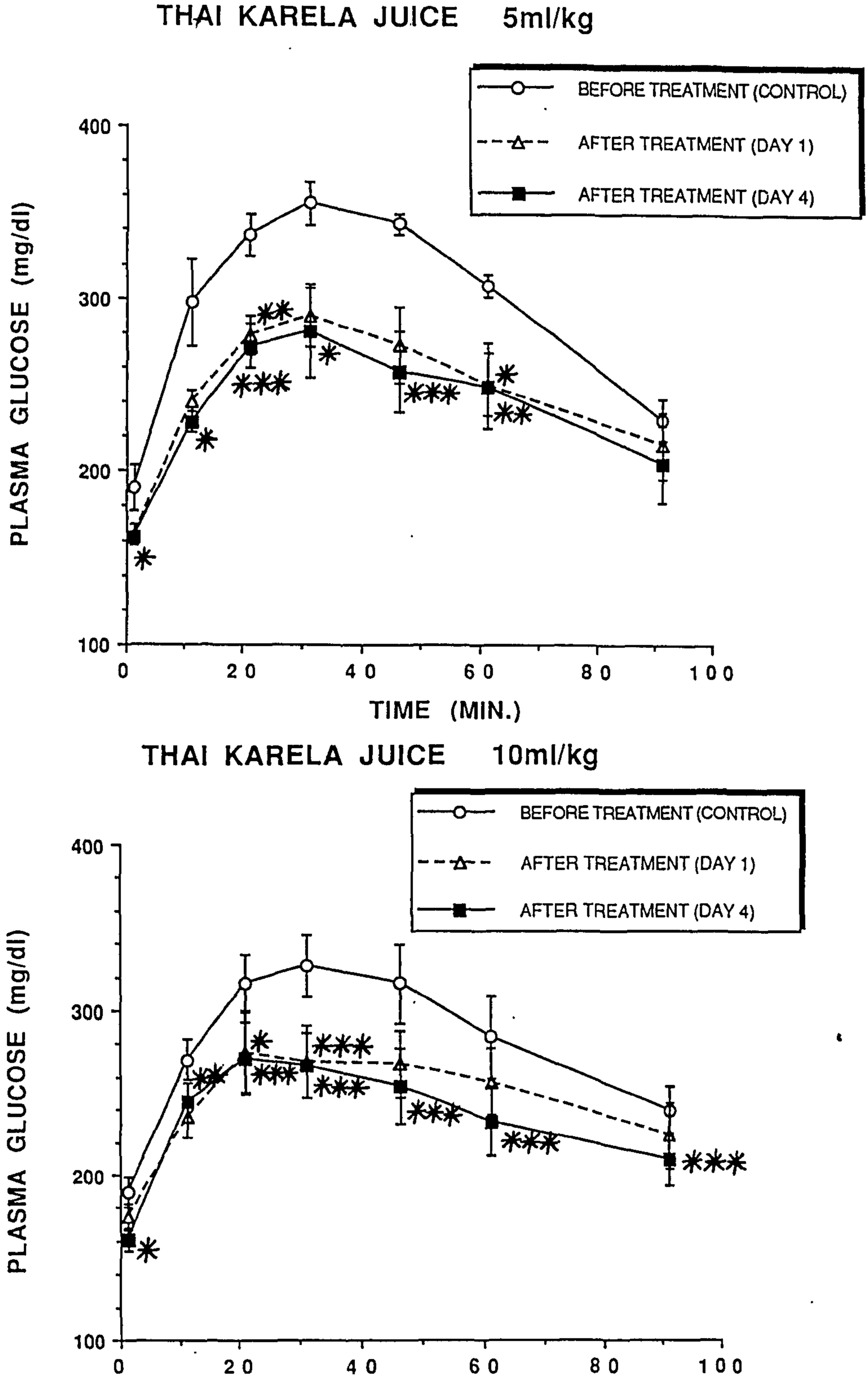


Figure 3.5: The dose-response effect of orally administered Thai/Kenyan karela juice (5, 10 or 15 ml/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=5 or 6). Glucose challenge was given at time 0; karela juice was given 30 min before glucose. Values of plasma glucose are mean ± SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control, by Student's paired t-tests.

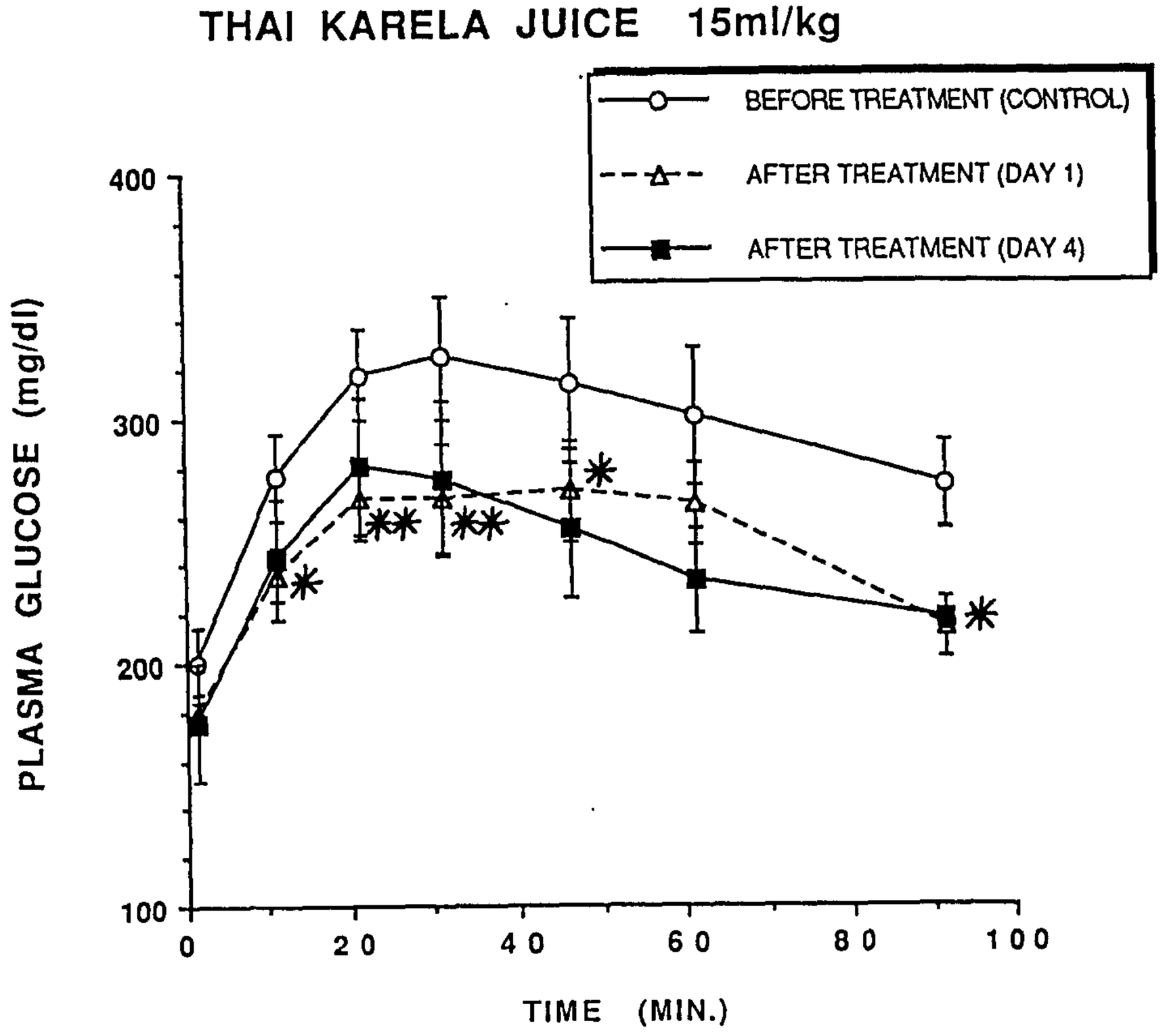
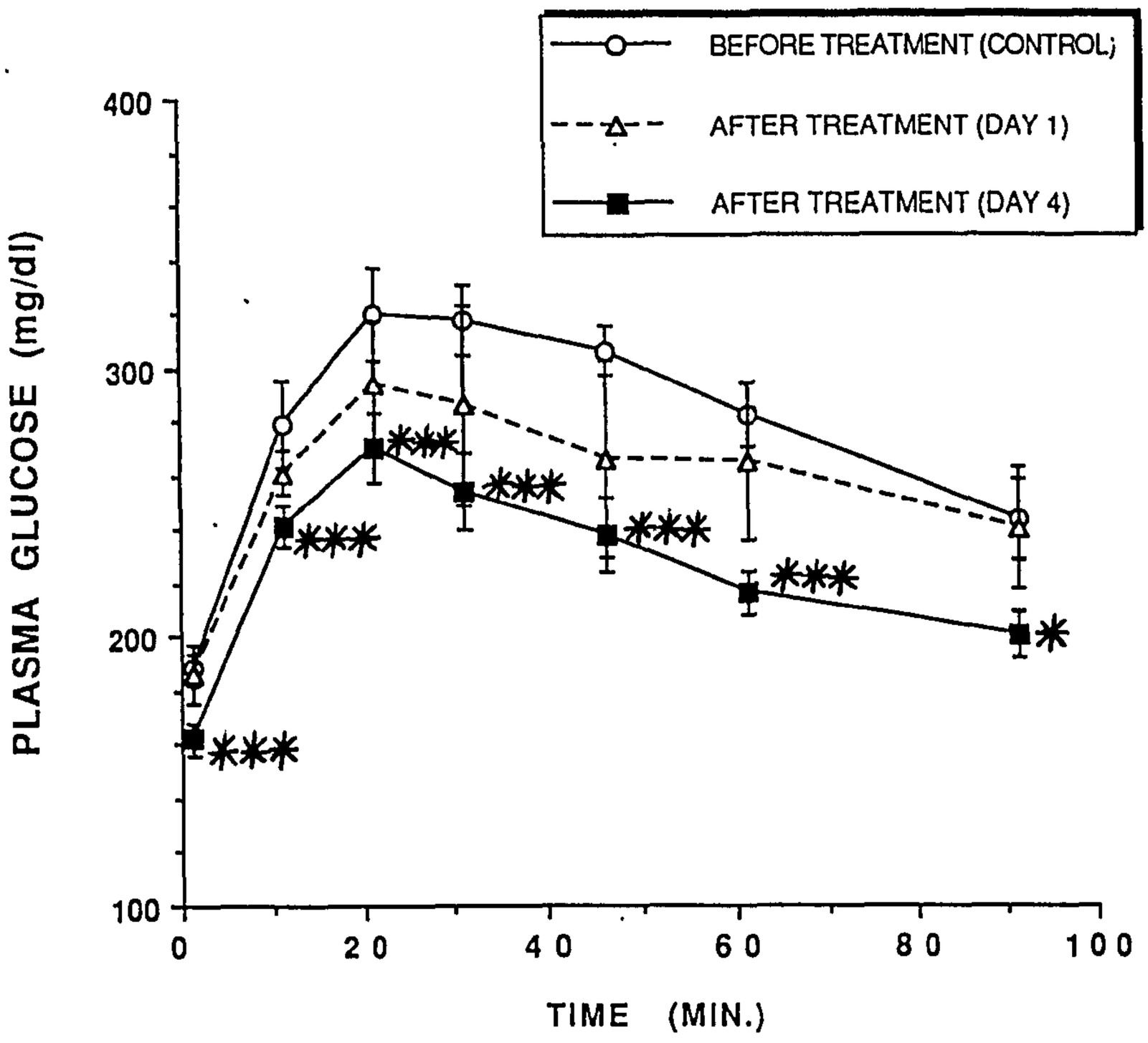

THAI KARELA JUICE 15ml/kg
BEFORE TREATMENT (CONTROL)
AFTER TREATMENT (DAY 1)
AFTER TREATMENT (DAY 4)
PLASMA GLUCOSE (mg/dl)
400
300
200
100
0
20
40
60
80
100
TIME (MIN.)

Figure 3.5 (continued):



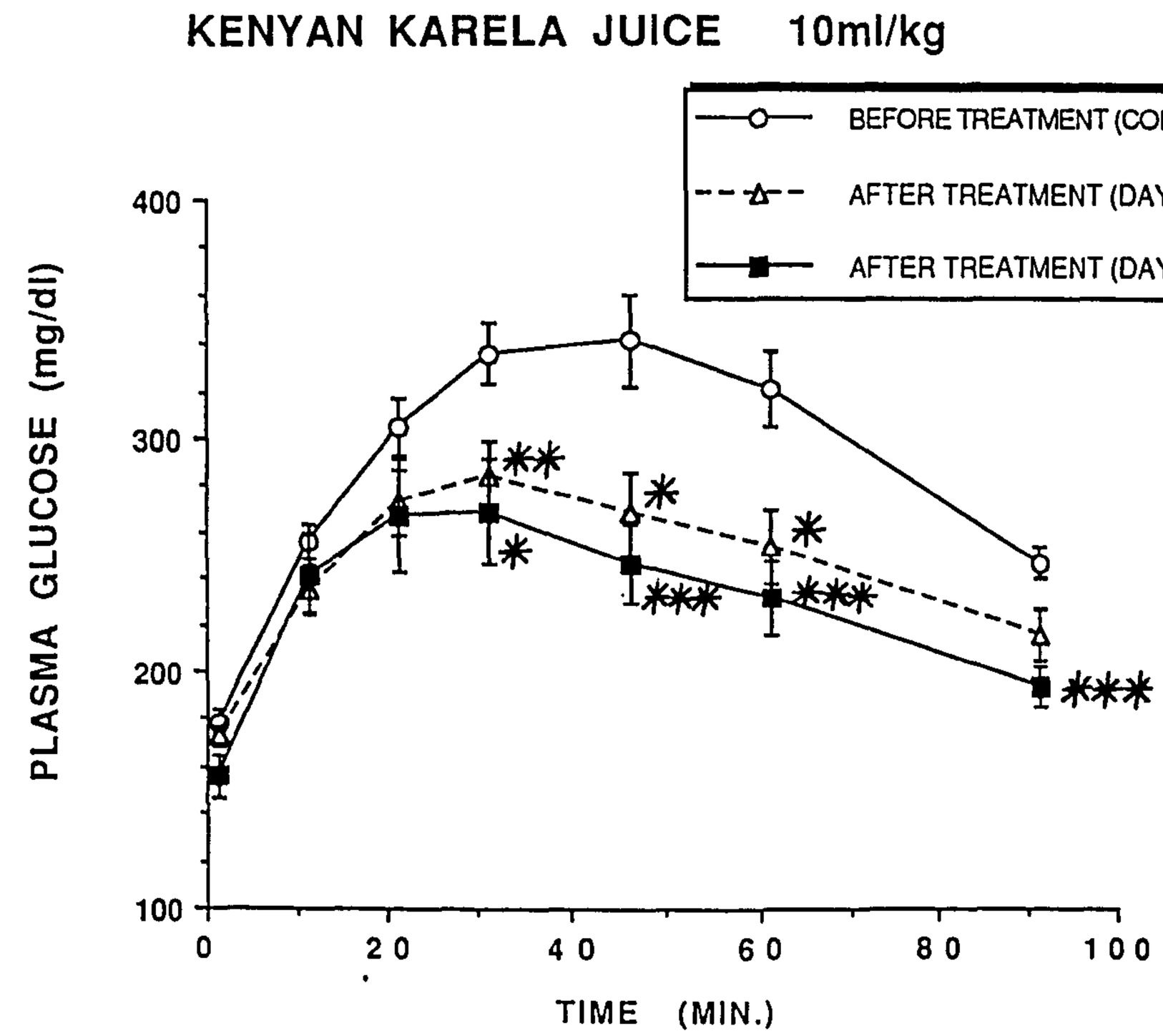


Figure 3.5 (continued):

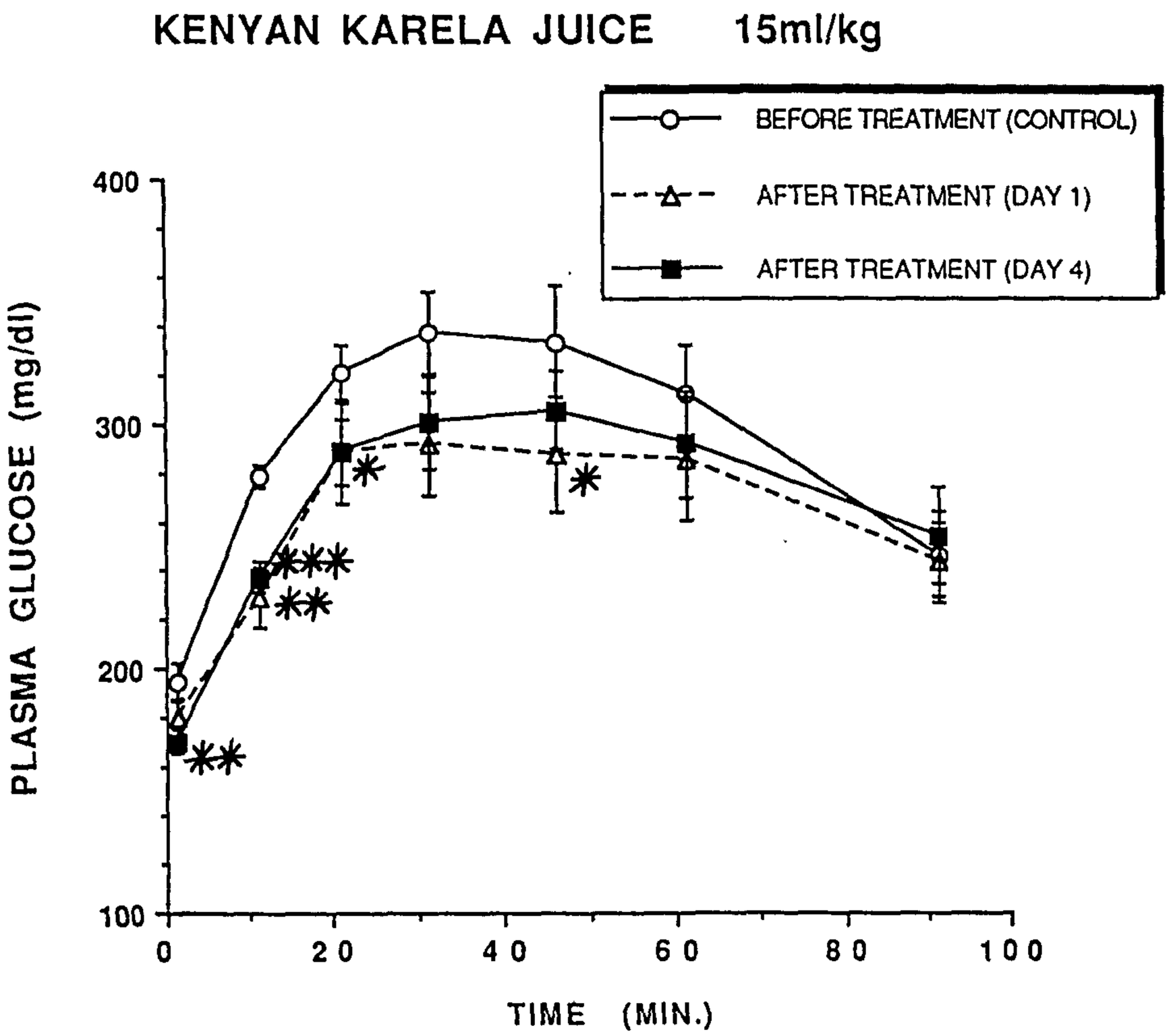


Table 3.7: Effect of orally administered karela (Thai or Kenyan; 5, 10 or 15ml/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; karela was given 30 min before glucose.

*p < 0.1, **p < 0.05, ***p < 0.02. ****p < 0.01 compared with control, by Student's paired t-tests. ΔG = the sum of the increase in plasma glucose at all time points compared with time 0.

Treatment	ΔG	
	mean ± SEM	% reduction
Thai karela		
Dose: 5ml/kg		
Control Day (n=6)	783.2 ± 81.4	-----
Day 1 (n=6)	564.2 ± 75.8	28.0
Day 4 (n=6)	523.5 ± 89.5 *	33.2 *
Dose: 10ml/kg		
Control Day (n=6)	614.8 ± 102.3	-----
Day 1 (n=6)	481.8 ± 82.8	21.6
Day 4 (n=6)	512.3 ± 68.0 *	16.7 *
Dose: 15ml/kg		
Control Day (n=5)	611.6 ± 75.4	-----
Day 1 (n=5)	446.4 ± 92.7	27.0
Day 4 (n=5)	460.8 ± 81.8 **	24.7 **
Kenyan karela		
Dose: 5ml/kg		
Control Day (n=6)	624.0 ± 87.0	-----
Day 1 (n=6)	499.3 ± 166.2	20.0
Day 4 (n=6)	414.3 ± 44.5 *	33.6 *
Dose: 10ml/kg		
Control Day (n=6)	740.3 ± 45.8	-----
Day 1 (n=6)	493.0 ± 73.5 ***	33.4 ***
Day 4 (n=6)	516.3 ± 62.8 ****	30.3 ****
Dose: 15ml/kg		
Control Day (n=6)	658.5 ± 109.5	-----
Day 1 (n=6)	545.2 ± 97.0 **	17.2 **
Day 4 (n=6)	654.3 ± 93.1	0.6

glucose but this was significant at only one time point, probably due to greater variation in values; however it resulted in a significant reduction in mean ΔG by 25% ($p = 0.041$). In addition, with all three dosages, the mean plasma glucose levels at 0 min on Day 4 were all lower than control and for two of them (at doses of 5 and 10ml/kg) the differences reached statistical significance.

For Kenyan karela juice, a single 5ml/kg dose improved mean values in the oral glucose tolerance curve but none of the time points reached statistical significance. However, the cumulative treatment with the same dosage resulted in significant reduction in plasma glucose at all time points and a reduction of mean ΔG by 34% ($p = 0.087$). When the dose was increased to 10ml/kg, the improvement in oral glucose tolerance was more pronounced in both acute and cumulative treatments, with a significant reduction in mean ΔG by 33% ($p = 0.016$) and 30% ($p = 0.006$) respectively. As the dose was further increased to 15ml/kg, instead of having a more pronounced improvement in oral glucose tolerance than that observed with the dose of 10ml/kg, both the acute and cumulative treatments improved oral glucose tolerance to a much smaller extent, and only the acute and not the cumulative treatment produced a significant reduction in mean ΔG (Table 3.7). However, the mean plasma glucose levels at 0 min on Day 4 were lower than control with all three dosages, with significant reduction at doses of 5 and 15ml/kg.

Plasma insulin

The effects of both Thai and Kenyan karela juice (at different dosages) on plasma insulin in n0 STZ diabetic rats, in response to an oral glucose load (2g/kg) are shown in Table 3.8. From the results obtained, a 5ml/kg single dose of Thai karela juice caused a decrease in plasma insulin at all time points, where the reduction in plasma insulin reached statistical significance at some time points. The cumulative administration of Thai karela juice (at the same dosage) resulted in a larger decrease in plasma insulin at most time points. As the dosage of Thai karela juice increased to 10 or 15ml/kg, similar results were obtained: both acute and cumulative administration resulted in reduction in plasma insulin at all time points, where cumulative administration had a more pronounced effect. However, the increase in

Table 3.8: Effect of orally administered Thai and Kenyan karela juice (5, 10 and 15ml/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; karela juice was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Thai karela							
Dose: 5ml/kg							
Control Day (n=6)	301.7 ± 43.1	328.0 ± 10.5	366.8 ± 37.7	369.0 ± 29.6	362.7 ± 39.6	327.2 ± 40.4	334.7 ± 36.9
Day 1 (n=6)	247.0 ± 28.3	262.3 ± 18.9**	319.5 ± 31.2	327.3 ± 40.8	304.8 ± 36.0	256.3 ± 29.1*	255.5 ± 15.8*
Day 4 (n=6)	218.3 ± 17.2	304.5 ± 31.5	297.7 ± 42.4	294.8 ± 27.1	232.0 ± 21.5*	227.7 ± 23.3*	211.7 ± 18.3***
Dose: 10ml/kg							
Control Day (n=6)	290.8 ± 29.1	422.0 ± 71.2	388.0 ± 35.2	377.8 ± 48.3	329.7 ± 42.4	361.3 ± 51.6	322.7 ± 22.4
Day 1 (n=6)	223.5 ± 32.5	307.7 ± 40.8	289.0 ± 37.2*	310.7 ± 48.9	320.0 ± 35.1	261.5 ± 32.8	255.7 ± 26.9
Day 4 (n=6)	205.2 ± 40.1	306.2 ± 49.0	270.5 ± 16.7*	256.2 ± 27.5	266.2 ± 25.2	222.3 ± 29.7	201.5 ± 26.3**

Table 3.8 (cont.): Effect of orally administered Thai and Kenyan karela juice (5, 10 and 15ml/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; karela juice was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Thai karela							
Dose: 15ml/kg							
Control Day (n=5)	376.4 ± 25.0	461.8 ± 58.8	505.4 ± 73.0	451.8 ± 63.2	385.4 ± 40.5	336.4 ± 21.1	324.2 ± 29.4
Day 1 (n=5)	311.2 ± 76.8	367.2 ± 87.5	371.8 ± 46.2*	392.8 ± 95.6	266.8 ± 39.9	324.8 ± 42.9	310.0 ± 57.6
Day 4 (n=5)	267.6 ± 37.4	334.0 ± 41.6	333.2 ± 20.3	366.4 ± 27.4	331.4 ± 51.9	284.4 ± 40.2	305.0 ± 51.5

Table 3.8 (cont.): Effect of orally administered Thai and Kenyan karela juice (5, 10 and 15ml/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; karela juice was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Kenyan karela							
Dose: 5ml/kg							
Control Day (n=6)	268.0 ± 35.3	354.8 ± 51.0	377.5 ± 40.8	324.7 ± 38.6	318.2 ± 27.3	320.8 ± 23.6	270.5 ± 25.4
Day 1 (n=6)	196.8 ± 21.9	370.2 ± 69.0	364.7 ± 50.0	329.3 ± 38.9	383.2 ± 55.7	302.7 ± 18.9	289.0 ± 40.5
Day 4 (n=6)	224.3 ± 22.6	322.3 ± 66.3	292.2 ± 23.0	285.7 ± 44.5	256.8 ± 29.2	238.0 ± 34.1***	228.0 ± 22.1
Dose: 10ml/kg							
Control Day (n=6)	300.3 ± 19.7	350.2 ± 31.9	404.5 ± 43.6	337.2 ± 28.4	329.8 ± 25.1	343.3 ± 27.5	340.8 ± 26.9
Day 1 (n=6)	256.7 ± 26.8*	344.3 ± 47.8	350.7 ± 47.9	311.7 ± 40.7	303.8 ± 47.8	375.5 ± 66.3	288.3 ± 35.8**
Day 4 (n=6)	218.0 ± 20.6***	320.7 ± 43.6	365.0 ± 89.8	308.0 ± 47.2	235.7 ± 51.0	259.0 ± 31.2**	208.8 ± 27.9**

Table 3.8 (cont.): Effect of orally administered Thai and Kenyan karela juice (5, 10 and 15ml/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; karela juice was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Kenyan karela							
Dose: 15ml/kg							
Control Day (n=6)	324.7 ± 32.3	350.3 ± 26.3	365.2 ± 64.0	338.8 ± 63.7	283.0 ± 27.1	275.8 ± 33.1	299.8 ± 43.1
Day 1 (n=6)	278.0 ± 63.9	261.7 ± 50.1	264.3 ± 67.9	282.0 ± 45.6	257.2 ± 46.5	261.3 ± 59.8	265.8 ± 42.9
Day 4 (n=6)	246.0 ± 37.0*	300.7 ± 36.4	285.2 ± 40.0	270.5 ± 40.2	273.8 ± 31.3	236.5 ± 31.3	241.8 ± 51.0

dosages of Thai karela juice did not produce a larger reduction in plasma insulin. Very similar results were obtained with different dosages of Kenyan karela juice.

3.3.3 Discussion

In this experiment, the dose-response effect of karela (both varieties) on oral glucose tolerance was examined. If the dosage and response are in a linear relationship, then one will expect that as the dose increases, a greater effect will be obtained. However, this seems not to be the case in this study. From the results, we observed that for both Thai and Kenyan karela, as the oral dose increased from 5ml/kg to 10ml/kg, the improvement on oral glucose tolerance became more significant. But as the dosage was further increased to 15ml/kg, the improvement on oral glucose tolerance appeared to be of a smaller extent than that of 10ml/kg. This suggests that 10ml/kg may be the optimum dose which can be used in this particular n0 STZ diabetic rat model. There are a few possible explanations for the reduced effect with the high dose of 15ml/kg. One explanation is that the volume used might be too large for the rats which caused diarrhoea and resulted in incomplete absorption of the dose (Kergoat, 1997). If this was the case, then we might have seen a loss in weight for the rats that were treated with the high dose of 15ml/kg during the four days of treatment. However, from the results (Appendix 2), weight reduction in rats treated with high dose (15ml/kg) of karela (both varieties) juice was not seen during the four days treatment period. Instead, there was a weight gain in both cases, though the weight gain was not statistically significant with high dose of Thai karela juice. Another possible explanation is that the amount of glucose (if present) in karela juice might have been absorbed, and so as the dose increased, more glucose (from karela juice) was absorbed which might have affected the results. By using quantitative HPLC (High Performance Liquid Chromatography), glucose was found to be present in both varieties of karela juice and the concentrations were ranged between 8-11mM and 7-8mM in Thai and Kenyan karela juice respectively (Appendix 6). From the calculations (Appendix 3), at a dose of 15ml/kg of karela juice, about 0.0095g extra glucose will be available for absorption. This is only a small quantity (about 1%w/w) as compared to the amount of glucose given in the oral glucose tolerance test (2g/kg, i.e. 0.7g for a rat weighing 350g). Therefore even if the glucose present in karela

juice was absorbed, it is unlikely to have affected the results of the oral glucose tolerance tests. However, on a closer examination of the results, the tailing effect with the high dose (15ml/kg) may not be a genuine effect. For instance, in the case of Thai karela juice,

Dose: 10ml/kg % reduction in mean $\Delta G = 21.6$ (Day 1); 16.7 (Day 4)

Dose: 15ml/kg % reduction in mean $\Delta G = 27.0$ (Day 1); 24.7 (Day 4)

a larger increase in % reduction in mean ΔG was observed between 10 and 15ml/kg dosages. However, in the case of Kenyan karela juice,

Dose: 10ml/kg % reduction in mean $\Delta G = 33.4$ (Day 1); 30.3 (Day 4)

Dose: 15ml/kg % reduction in mean $\Delta G = 17.2$ (Day 1); 0.6 (Day 4)

a sharp decrease in % reduction in mean ΔG was obtained between 10 and 15ml/kg dosages. In addition, tolerance might have developed which explained the Day 4 result at dose of 15ml/kg. Thus the dose/response may differ with the variety of karela tested.

Apart from the above, the basal glycaemia (plasma glucose at time 0, just before the glucose challenge) was always lowered on Day 4, and in some cases, it reached statistical significance (Fig. 3.5). This suggests that both varieties of karela had an overall improvement in hyperglycaemia. This was postulated earlier (Section 3.2.3).

It is surprising to note that the initial (phase I) stimulation of insulin by karela (which was reported earlier in Section 3.2) was not observed here (Table 3.8), despite the fact that same experimental conditions were used. Instead of a rise in plasma insulin, both varieties of karela, at all different dosages, caused a decrease in insulin levels at all time points. These results are contradictory to what we observed earlier (Fig. 3.4). However, a detailed examination of the data obtained suggested that the insulin results may be unreliable, due to the fact that acute and cumulative treatment with both water and metformin (as controls) also resulted in a decrease in plasma insulin at most time points (Table 3.9). Another possible explanation is that different batches of karela were used in these experiments which may account for the variations in results.

Table 3.9: Effect of orally administered water (10ml/kg body weight) and metformin (200mg/kg body weight; 200mg in 10ml water) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; water or metformin was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Water							
Control Day (n=5)	215.4 ± 23.1	311.0 ± 24.0	325.4 ± 37.2	314.4 ± 34.4	280.6 ± 27.9	253.2 ± 23.0	286.6 ± 44.8
Day 1 (n=5)	199.2 ± 27.3*	304.2 ± 41.4	326.6 ± 52.8	252.0 ± 35.1	225.4 ± 41.6	186.0 ± 48.8	236.8 ± 40.2
Day 4 (n=5)	168.8 ± 14.1	244.6 ± 29.6*	228.2 ± 38.7*	238.6 ± 14.5	226.6 ± 33.8	239.0 ± 70.3	196.6 ± 20.9*
Metformin							
Control Day (n=6)	324.2 ± 30.4	431.7 ± 54.9	394.2 ± 37.9	357.5 ± 40.4	368.2 ± 54.7	397.8 ± 41.7	330.7 ± 30.6
Day 1 (n=6)	241.0 ± 44.6	360.2 ± 47.0*	352.8 ± 37.3	342.8 ± 31.2	347.8 ± 60.8	303.3 ± 46.0*	283.7 ± 34.8
Day 4 (n=6)	223.7 ± 29.8***	347.0 ± 52.7**	368.8 ± 56.1	316.0 ± 32.9*	260.7 ± 37.5	253.7 ± 41.6***	218.7 ± 24.8***

Further experiments are required in order to confirm the insulin-stimulating effect of karela. However, the present results suggested that the anti-hyperglycaemic effect of karela was most likely caused by mechanisms other than insulin stimulation.

This study indicates that the dose and the variety of karela tested may affect the results of the oral glucose tolerance obtained. In addition, the dose-response relationship for karela was not linear, i.e. increase in dosage did not always result in an increase in response. This study is believed to be one of the first detailed investigation on the dose-response effect of two varieties (Thai and Kenyan) of karela on oral glucose tolerance using a diabetic animal model, though various dosages of karela had been tested with regard to its anti-diabetic effect by previous researchers on separate occasions in diabetic animals (Raman and Lau, 1996). For example, Karunanayake *et al.* (1984) studied the effect of karela juice (0.25, 0.5 and 1ml/100g body weight of Sprague-Dawley rat) on glucose tolerance and the variation of effect with dose was noted.

3.4 Effect of whole karela juice, the supernatant and the sediment on oral glucose tolerance

It was found that solids in fresh Thai karela juice (prepared as described in Section 3.2.1) sedimented out on standing to give a clear liquid on top and fine residue at bottom. As a result, an oral glucose tolerance test was carried out to see if the anti-hyperglycaemic activity resided in the supernatant or the sediment.

3.4.1 Method

The experimental procedures for the oral glucose tolerance test were the same as before. The four different treatment groups were: water (10ml/kg body weight) as a control, whole karela juice (10ml/kg), supernatant (10ml/kg) and sediment (10ml/kg). The supernatant and sediment were separated by centrifuging the whole juice. The sediment was resuspended in water before administration. The dosages of both supernatant and sediment used in the test were equivalent to 10ml/kg of whole karela juice. The different treatments were given for four consecutive days and the blood sampling was carried out on Test Day 4.

3.4.2 Result and discussion

From the results (Fig. 3.6), the cumulative effects (after four days of treatment) of both the supernatant and the sediment improved oral glucose tolerance to a very similar extent, though in both cases, the plasma glucose level at all individual time points did not reach statistical significance. However, the effects of the supernatant and the sediment were only half of that produced by the whole juice, in which there was a significant decrease in plasma glucose at most time points. These results suggested that the anti-diabetic compound(s) was present in both the supernatant and sediment (may be in very similar concentration) and therefore the whole juice was used for future testing.

3.5 Investigation of karela juice solvent extracts on oral glucose tolerance

From the results obtained so far, it was shown that whole karela juice of Thai variety, at dosage of 10ml/kg, had the most promising improvement on oral glucose tolerance in n0 STZ NIDDM model. Thus attempt was made to fractionate the whole juice in order to further study the active ingredient(s) and the mode of action.

3.5.1 Materials and methods

Preparation of karela extracts

Freshly prepared karela juice (Thai variety only) was frozen immediately (after preparation as in Section 3.2.1) and later freeze-dried. The freeze-dried juice (approximately 30g dry weight equivalent to 1 litre juice) was sequentially extracted (using Soxhlet extraction) with solvents (of increasing polarity) in the following order: hexane, chloroform, methanol and water, for 6 hours each time. Each extract was evaporated to dryness using a rotary vacuum evaporator. Figure 3.7 showed the TLC zone profiles of the constituents of the different extracts, apart from the water extract in which no suitable solvent system was yet found to move the extract up the baseline. As shown on the TLC plates, the three different extracts contained different constituents. From the sequential solvent extraction, one would expect the most non-polar compounds to be extracted in the hexane extract, whereas the most polar compounds would be retained in the water extract.

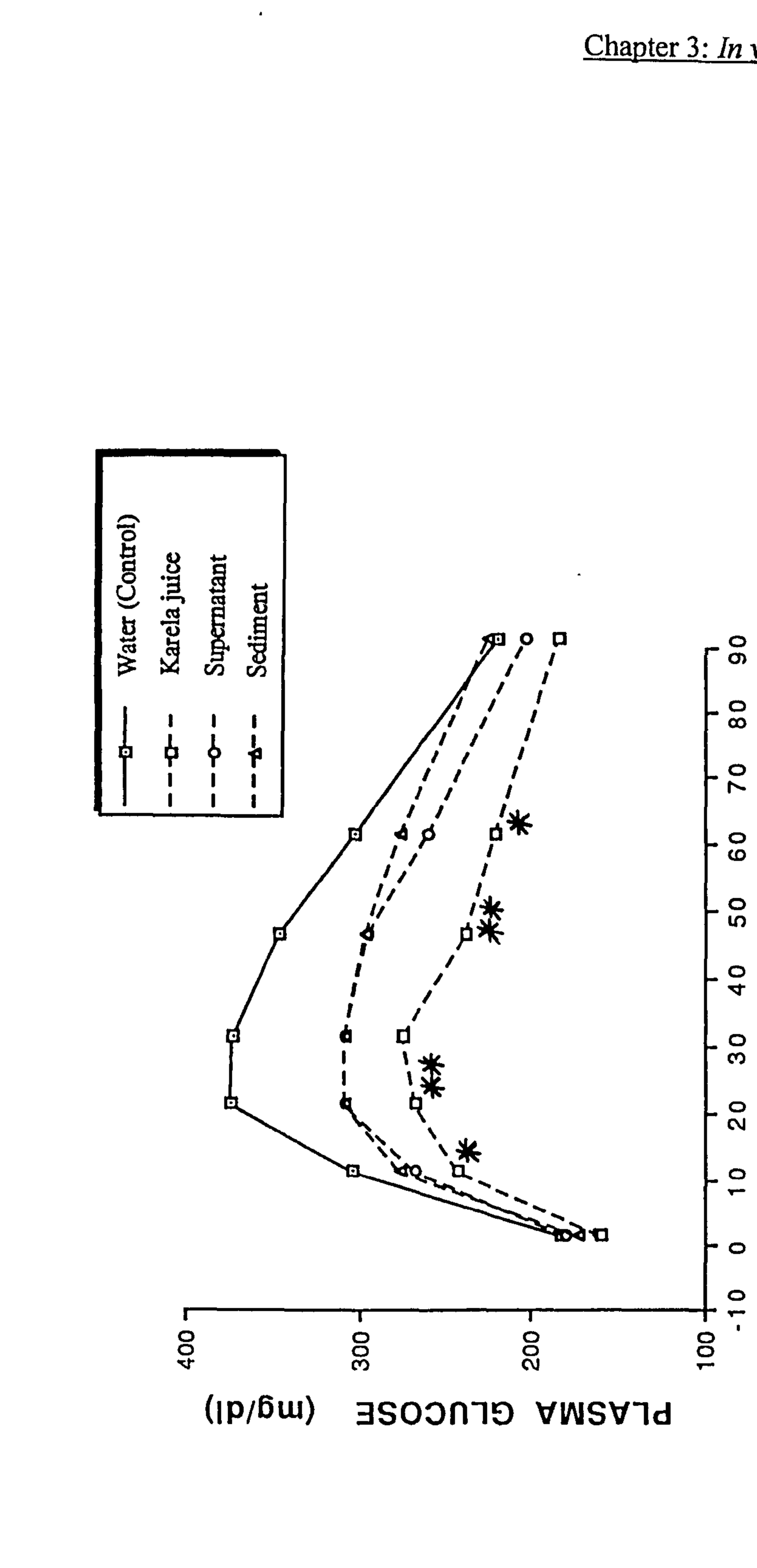


Figure 3.6: Effect of orally administered Thai karela juice, supernatant and sediment (10ml/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=5) after four days of treatment.

Mean plasma glucose values are plotted.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ compared with control, by Student's unpaired t-tests.

N.B. For the sake of clarity, vertical bars representing standard error of mean are not shown on graph.

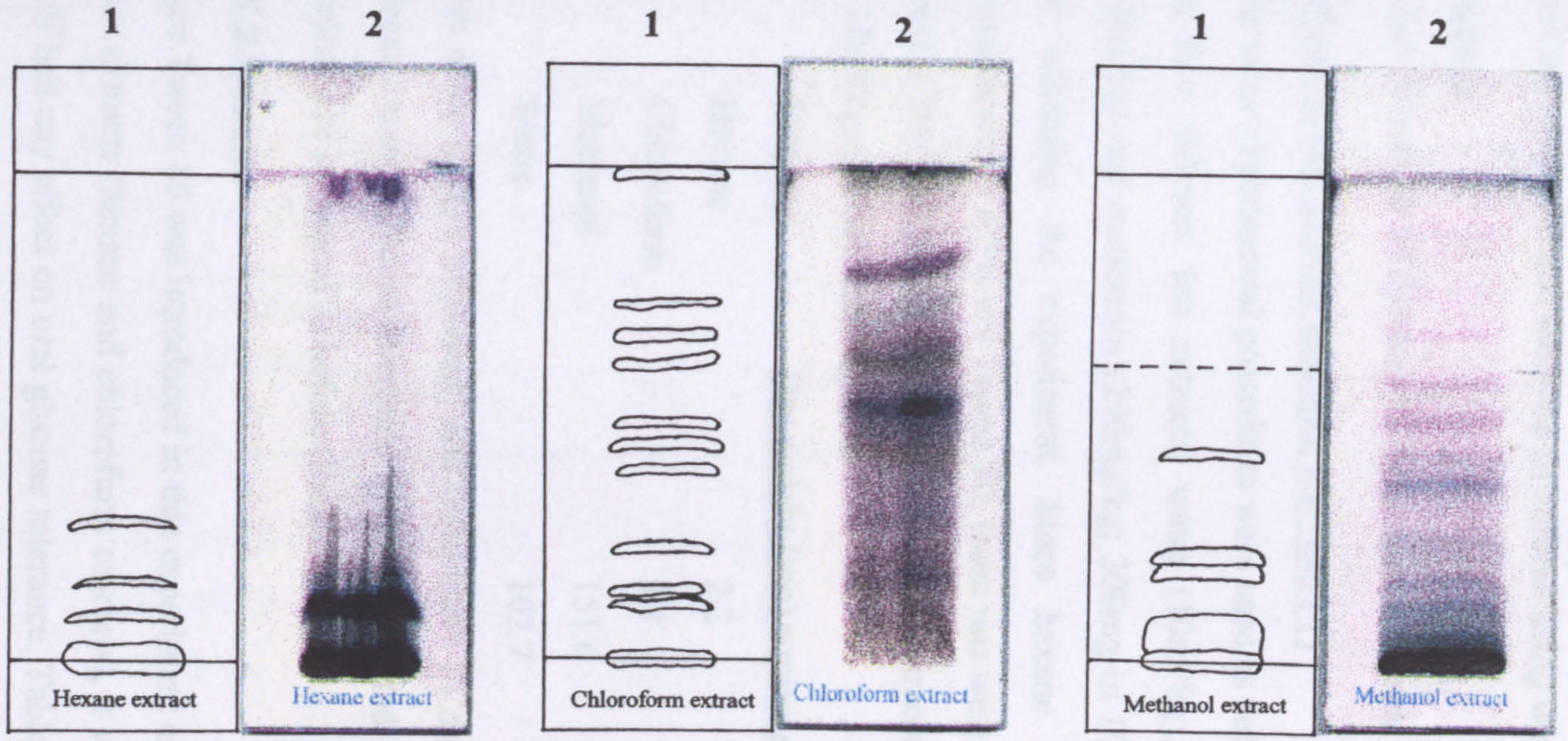


Figure 3.7: TLC zone profiles of the three extracts of Thai karela juice

10µl of each extract was spotted on silica gel GF_{254} plate. Different solvent systems were used according to the polarity of the extract:

Hexane extract – toluene: chloroform = 1:1

Chloroform extract – chloroform: ethanol = 12:1

Methanol extract – chloroform: ethanol = 12:1 up to the marked solvent front; followed by chloroform: ethanol = 10:1 half way up the plate.

Keys: 1 – Under UV light (λ = 254nm)

2 – With anisaldehyde spraying agent

For *in vivo* studies, residues were made up to the equivalent volume of juice from which they were extracted. Hexane and chloroform extracts were each reconstituted in 0.3% v/v Tween 80 in water (since these extracts are insoluble in water and Tween 80 acts as a solublising agent (Martindale, 1996)). Methanol and water extracts were each reconstituted and made up to volume using water since they were water soluble.

Animals

Adult Wistar n0 STZ induced NIDD rats were used as before.

Effects on oral glucose tolerance test (OGTT)

The same experimental procedures were used as before (Section 3.2.1). In addition to the four different test extracts, water (10ml/kg), 0.3% v/v Tween 80 in water (10ml/kg) and metformin (200mg/kg; 200mg in 10ml water) were used as controls for validating the experiment. Since hexane and chloroform extracts were reconstituted in 0.3% v/v Tween 80, those rats were given Tween instead of water on Control Day (Appendix 4). The dosages of extracts used in the test were equivalent to 10ml/kg of whole karela juice:

Extracts	Dry weight (mg) equivalent to 10ml of whole karela juice
Hexane	2.2
Chloroform	9.8
Methanol	151.6
Water	102.7

Both acute (single treatment) and cumulative (4 days treatment) effects of the four extracts on oral glucose tolerance were investigated. Both plasma glucose and plasma insulin were measured as before (Section 3.2.1).

3.5.2 Results

Since Tween 80 was introduced in this experiment as a solublising agent for the non-polar extracts (hexane and chloroform extracts), it was necessary to check if Tween itself had any effect on oral glucose tolerance. Table 3.10 shows the results with the three controls: water, metformin and 0.3% v/v Tween 80. As expected, water (as a negative control) had no significant effect on oral glucose tolerance, whereas metformin (a positive control) exhibited a significant improvement in oral glucose tolerance on both acute and cumulative treatment. In the case of 0.3% v/v Tween 80,

Table 3.10: Effect of orally administered water (10ml/kg body weight), metformin (200mg/kg body weight; 200mg in 10ml water) and 0.3%v/v Tween 80 in water (10ml/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; water, metformin or Tween 80 was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Water							
Control Day (n=5)	208.0 ± 17.0	314.6 ± 21.8	354.8 ± 40.5	381.8 ± 51.6	381.6 ± 56.3	333.2 ± 52.7	231.6 ± 30.4
Day 1 (n=5)	224.2 ± 14.9	330.8 ± 22.3	372.0 ± 31.1	368.0 ± 43.4	375.0 ± 45.8	335.2 ± 41.5	243.6 ± 24.7
Day 4 (n=5)	166.0 ± 4.8	258.8 ± 21.7	309.6 ± 32.9	314.8 ± 41.2	339.6 ± 38.9	309.4 ± 40.0	253.8 ± 25.8
Metformin							
Control Day (n=5)	253.0 ± 22.2	362.8 ± 43.5	418.0 ± 54.4	432.4 ± 60.5	400.2 ± 55.0	359.4 ± 55.6	302.0 ± 44.3
Day 1 (n=5)	229.8 ± 22.6	258.2 ± 24.0**	265.0 ± 22.8**	262.2 ± 26.8**	253.6 ± 16.4*	242.8 ± 24.2*	216.2 ± 27.5
Day 4 (n=5)	169.8 ± 20.8*	210.0 ± 28.3*	225.5 ± 31.3*	227.5 ± 29.9*	239.3 ± 35.1*	239.0 ± 35.2	210.5 ± 31.6
0.3%v/v Tween 80							
Control Day (n=5)	225.5 ± 14.0	344.5 ± 27.9	374.8 ± 32.6	389.8 ± 30.3	341.8 ± 43.8	315.8 ± 33.4	244.0 ± 23.4
Day 1 (n=5)	220.8 ± 27.1	326.3 ± 40.9	350.3 ± 54.5	331.8 ± 53.9	323.0 ± 51.4	292.0 ± 50.8	217.5 ± 17.3
Day 4 (n=5)	196.8 ± 21.2	310.0 ± 22.3	342.0 ± 22.1	333.0 ± 44.1	327.5 ± 38.8	287.3 ± 55.2	244.0 ± 27.7

which acts as a negative control, a slight improvement in oral glucose tolerance was observed, though the reduction of plasma glucose at all time points did not reach statistical significance on both Days 1 and 4. These results validated our experimental model.

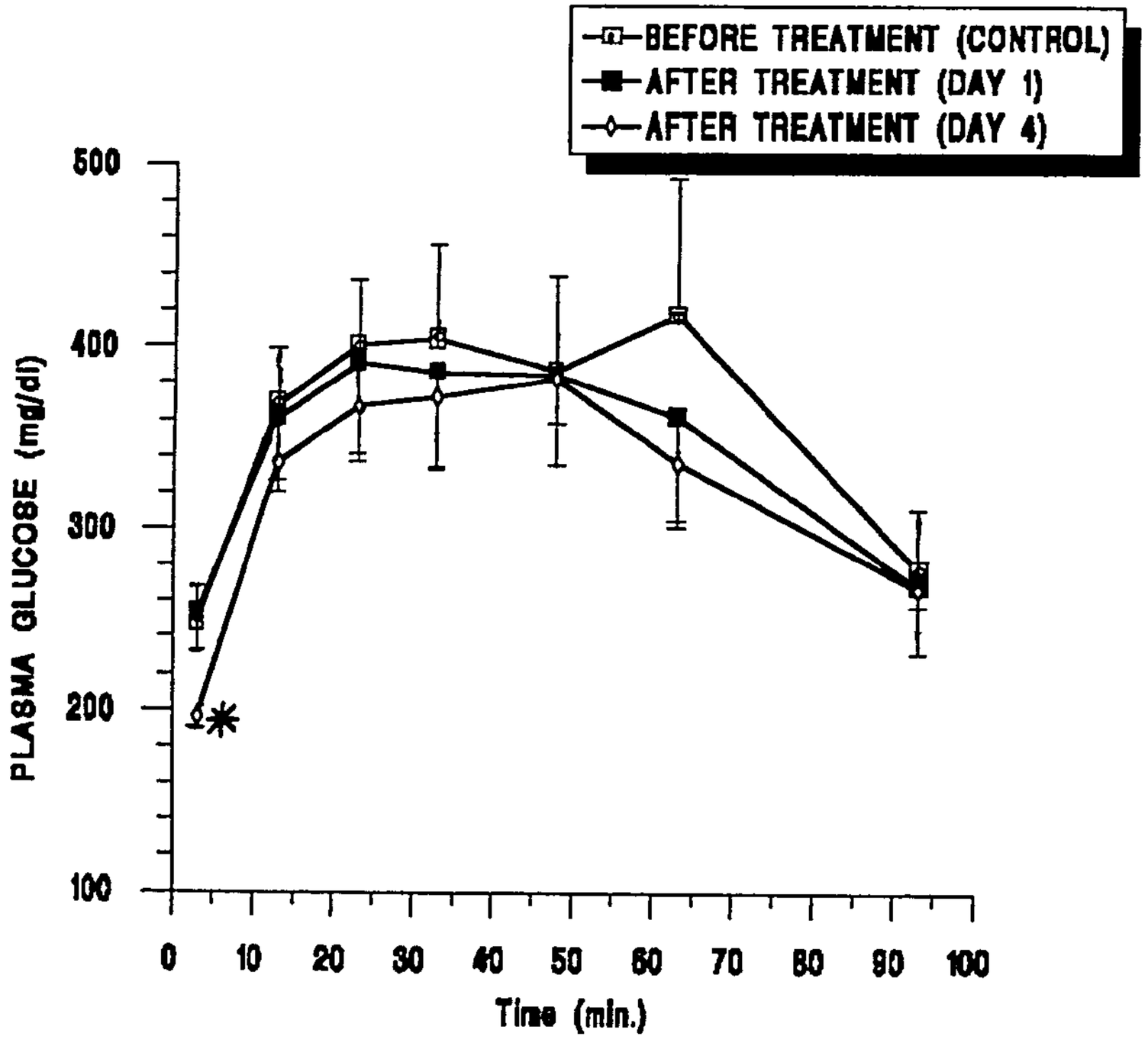
From the results of the test extracts (Fig. 3.8), among the four different karela extracts, only the hexane and water extracts significantly improved oral glucose tolerance, where a more pronounced effect was found on Day 4 (as compared to Day 1) in both cases. Both acute and cumulative treatment with chloroform extract did not improve oral glucose tolerance. In addition, it is interesting to note that the methanol extract had a tendency of increasing plasma glucose at most time points on Day 1, though the differences did not reach statistical significance. On Day 4, there was a slight decrease in mean plasma glucose at most time points, though again this did not reach statistical significance.

The effect of all four extracts on plasma insulin in response to the oral glucose load was shown in Fig. 3.9. The results showed that both hexane and water extracts, after acute or cumulative administration, caused a rise in mean plasma insulin at 10 min time point (phase I release, Section 1.1.7), though the rise in all cases did not reached statistical significance. However, this phase I insulin release was not observed with both the chloroform and methanol extracts, though the plasma insulin level at time 0 on both Days 1 and 4 was significantly raised with the chloroform extracts.

3.5.3 Discussion

In the previous chapter, the TLC zone profile (Fig. 2.13) of the whole Thai karela juice revealed that it contained many compounds. In order to find out which compound(s) is responsible for the anti-hyperglycaemic effect, it was necessary to fractionate the whole juice. Here, a sequential soxhlet extraction using solvents of different polarities was employed for the initial fractionation of the whole juice. This method aims to extract different groups of compounds into different solvents, according to their polarities (as shown in Fig. 3.7). In this case, one expected the most non-polar compounds would have been extracted by hexane, then slightly more polar compounds would have been extracted by chloroform, followed by methanol, and finally the most polar compounds would go into the water extract. However, it is

a)



b)

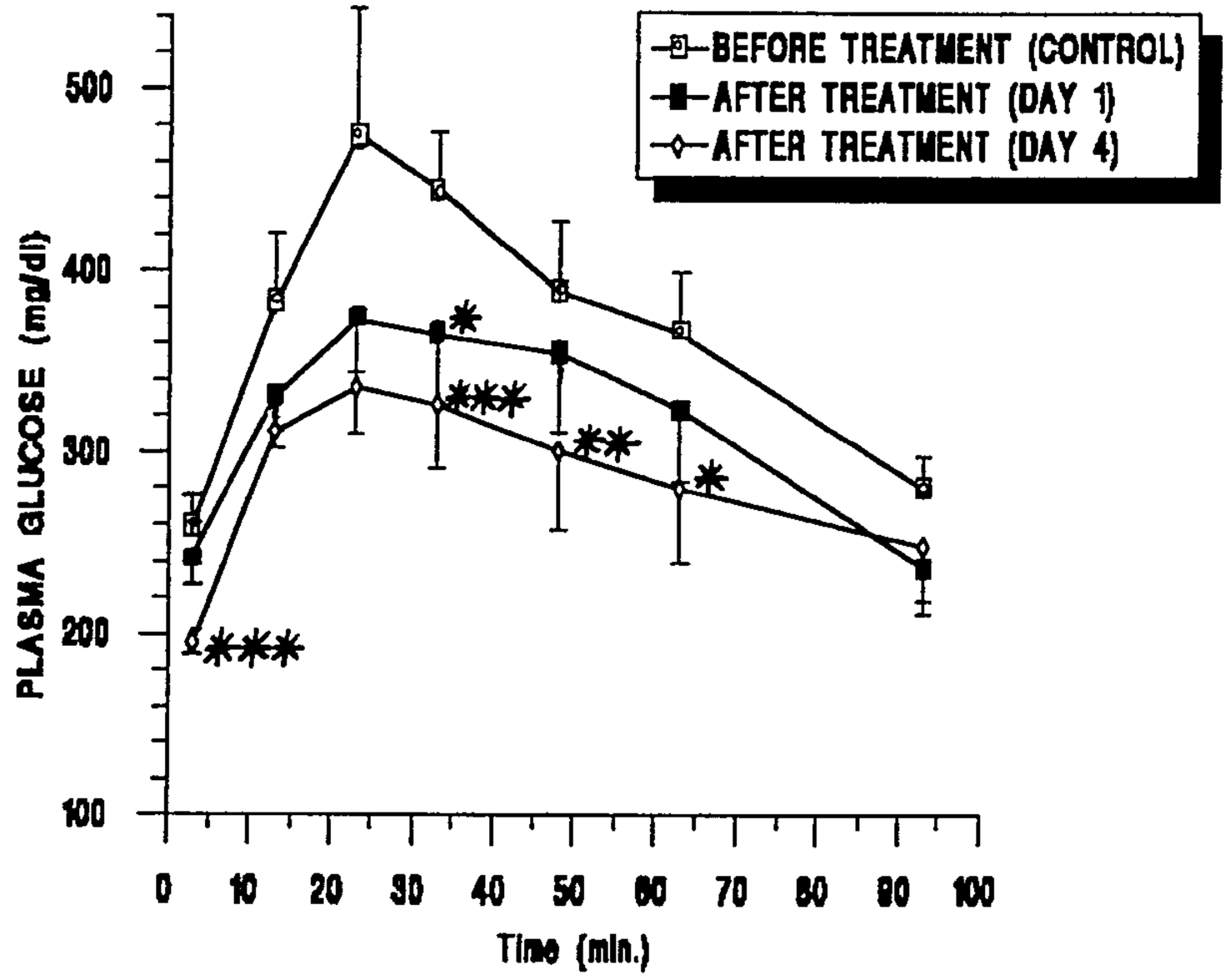
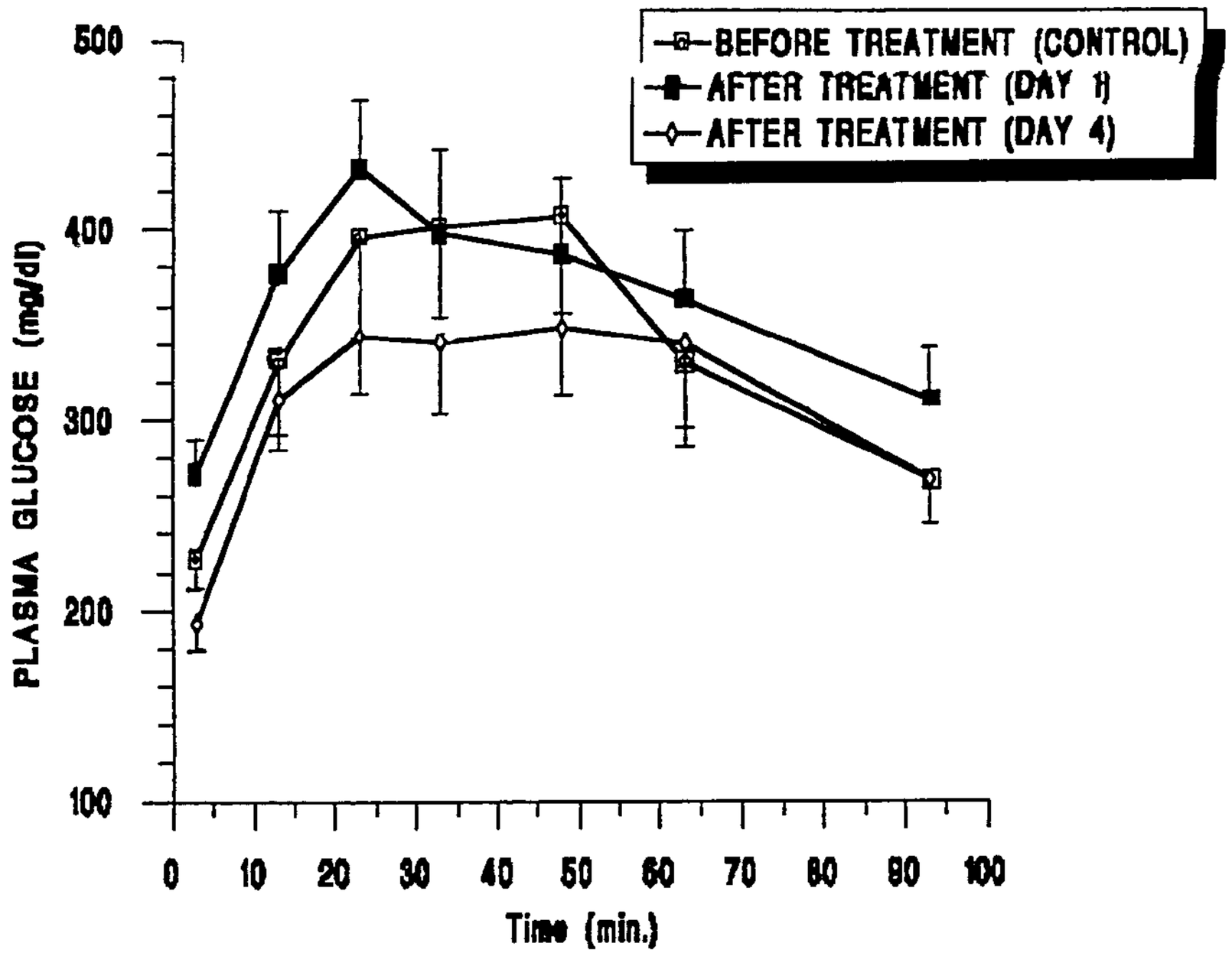


Figure 3.8: Effect of orally administered *a) Chloroform extract* (10ml(9.8mg)/kg body weight) and *b) Hexane extract* (10ml(2.2mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=5). Glucose challenge was given at time 0; karela extracts were given 30 min before glucose. Values of plasma glucose are mean +/- SEM.

* $p < 0.05$, ** $p < 0.02$, ***$p < 0.01$ as compared with control

c)



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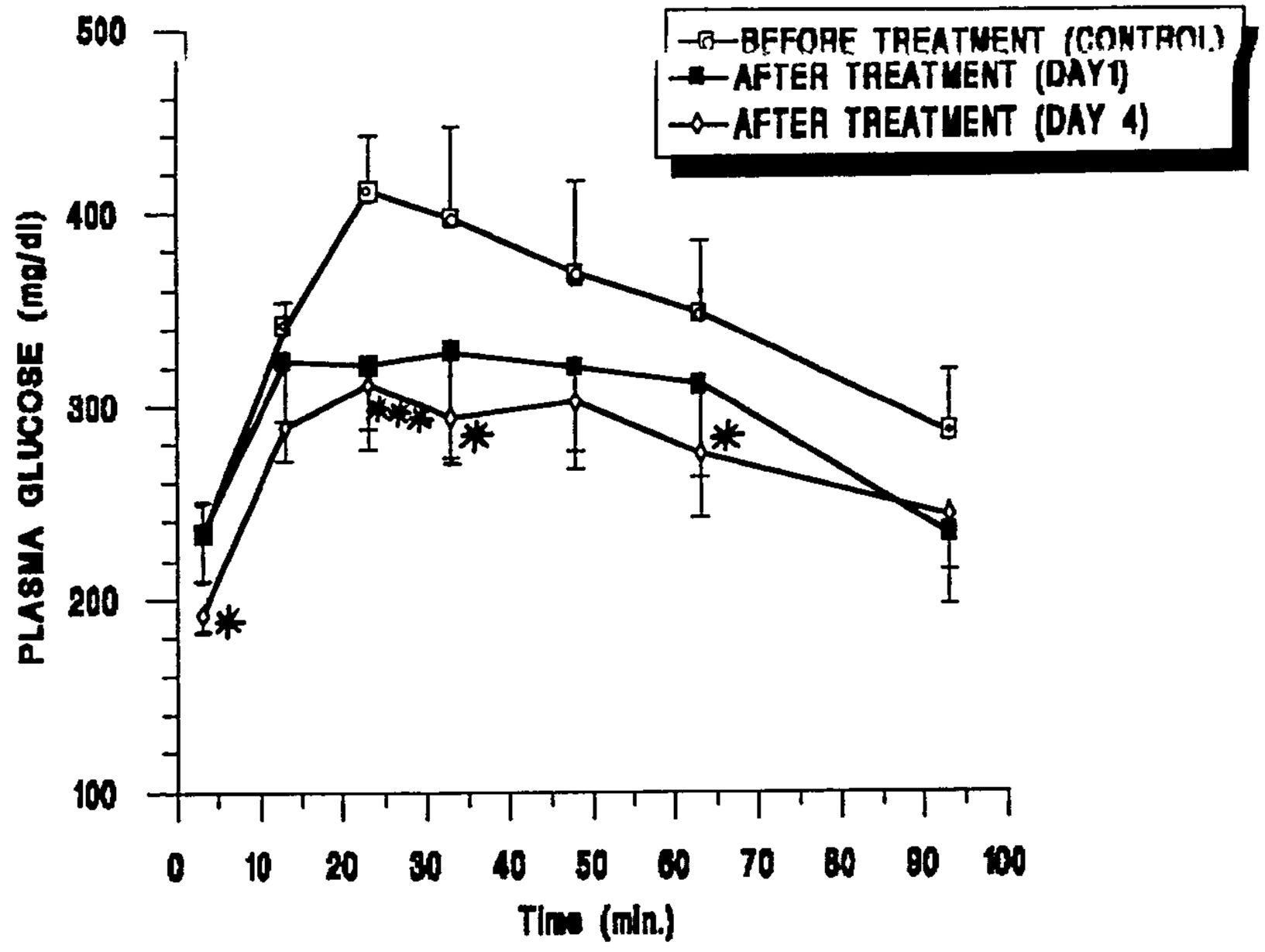
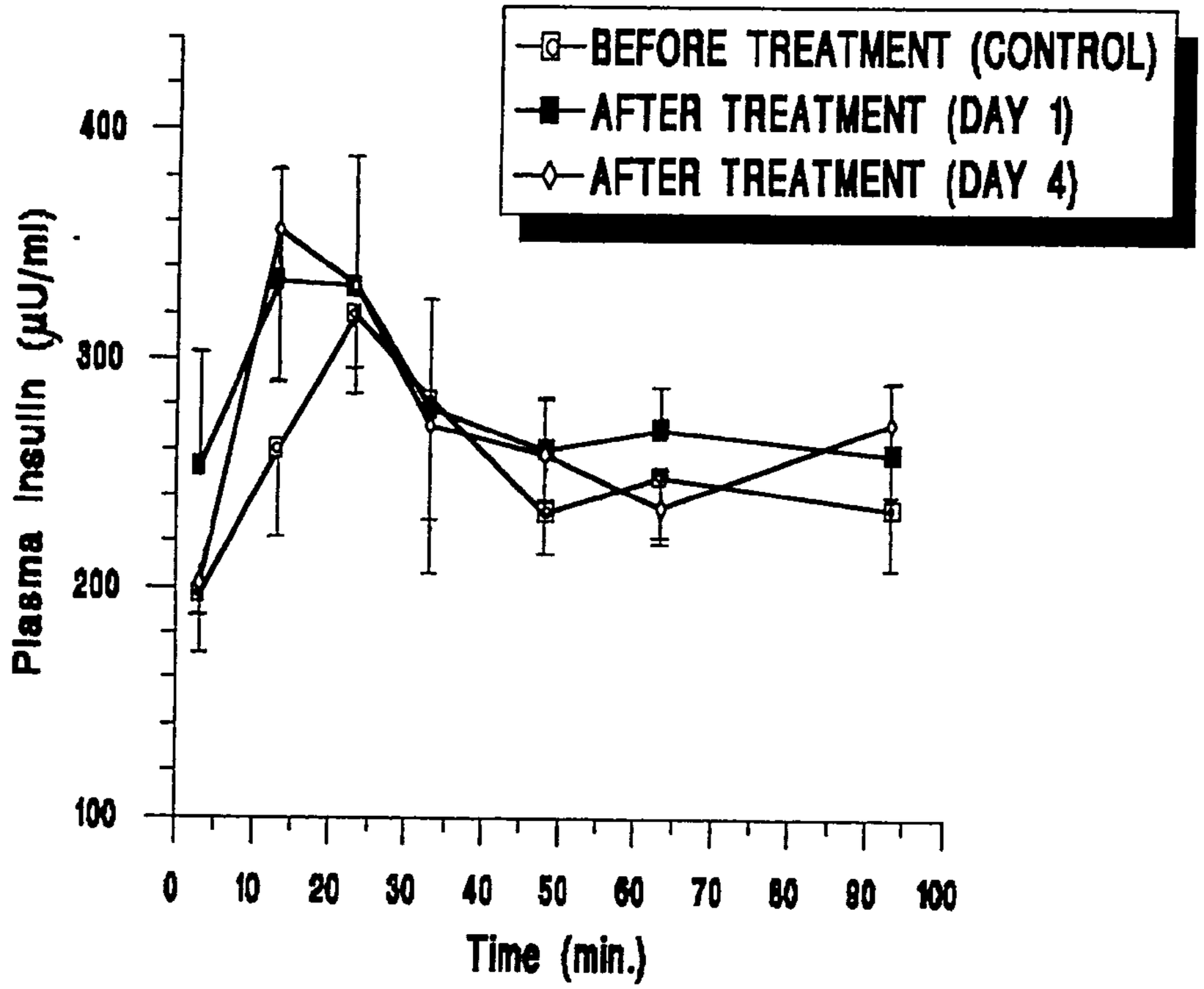


Figure 3.8 (cont.): Effect of orally administered *c) Methanol extract* (10ml(151.6mg)/kg body weight) and *d) Water extract* (10ml(102.7mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=5). Glucose challenge was given at time 0; karela extracts were given 30 min before glucose. Values of plasma glucose are mean +/- SEM.

* $p < 0.05$, ** $p < 0.02$, ***$p < 0.01$ as compared with control

a)



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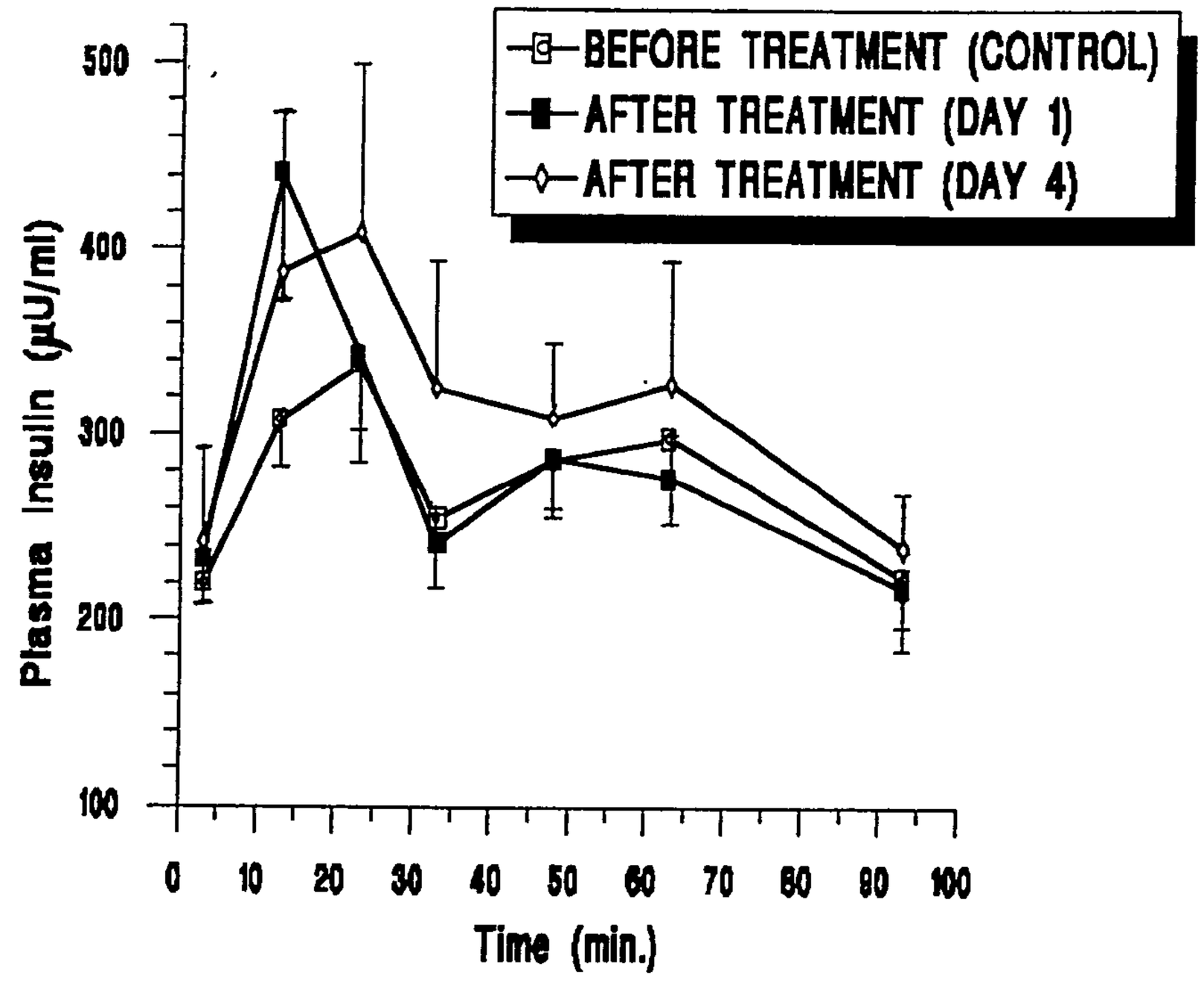
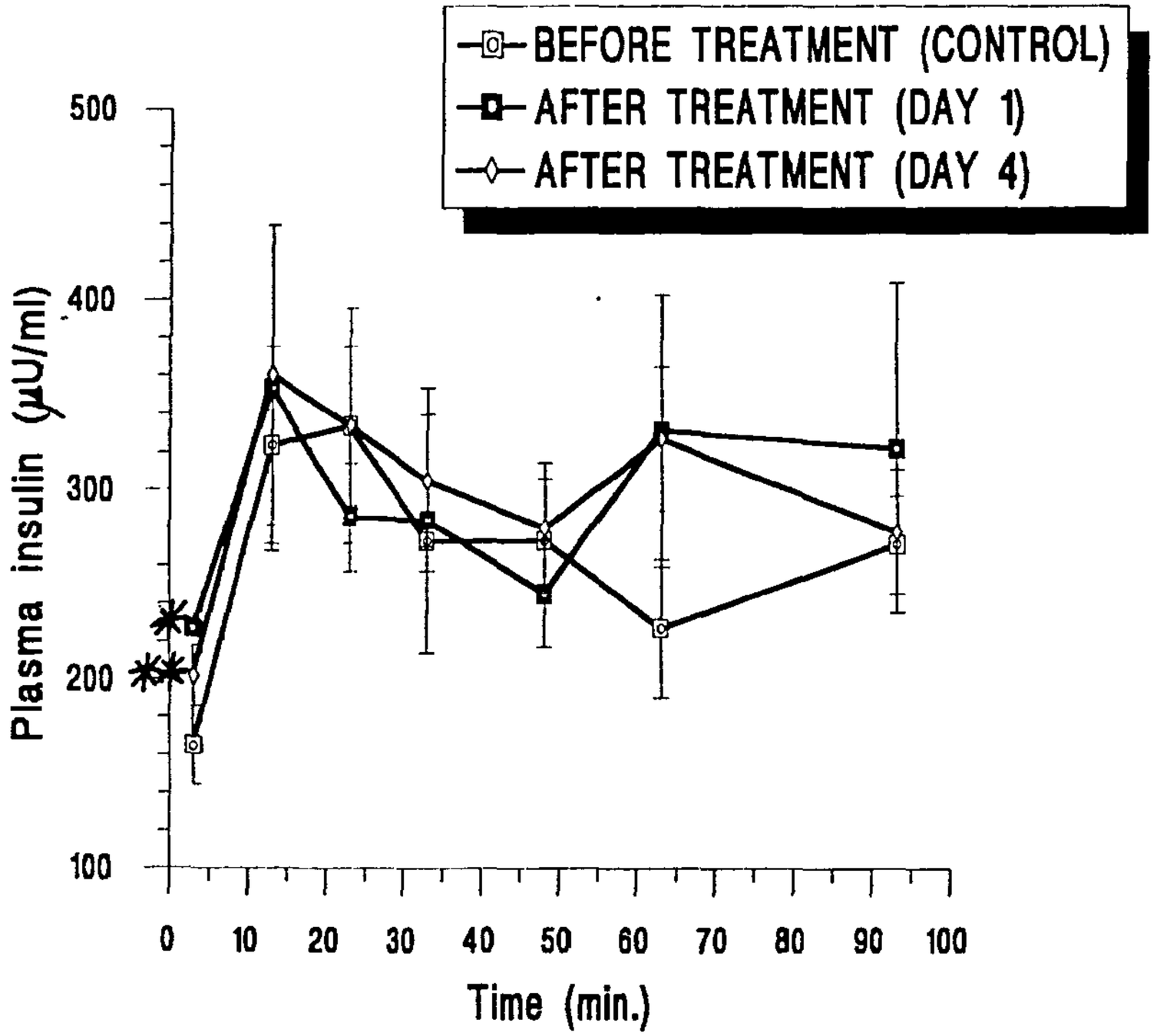


Figure 3.9: Effect of orally administered *a) Hexane extract* (10ml(2.2mg)/kg body weight) and *b) Water extract* (10ml(102.7mg)/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min. before glucose. Values of plasma insulin are mean +/- SEM.

c)



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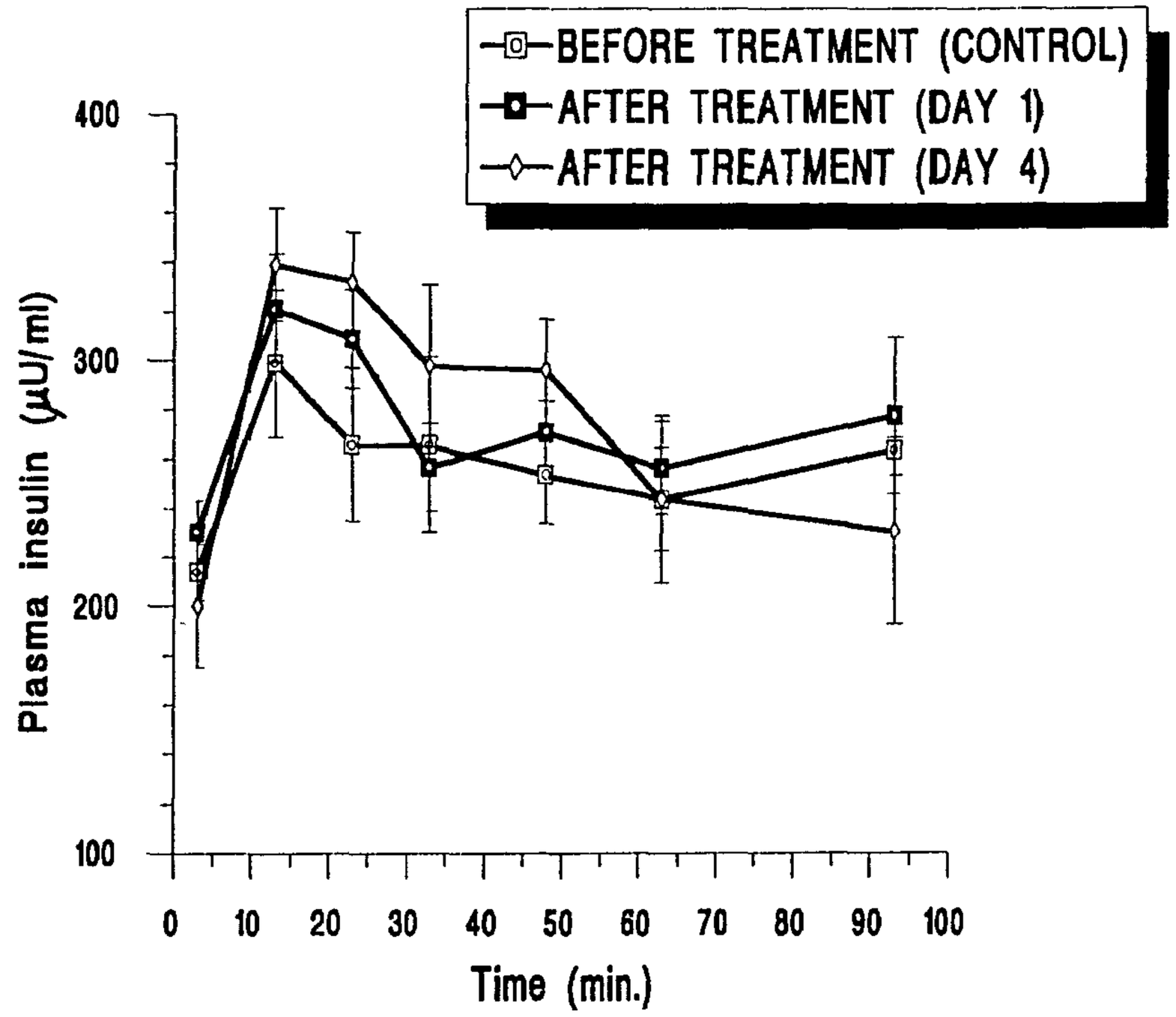


Figure 3.9 (cont.): Effect of orally administered *c) Chloroform extract* (10ml(9.8mg)/kg body weight) and *d) Methanol extract* (10ml(151.6mg)/kg body weight) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min. before glucose. Values of plasma insulin are mean +/- SEM. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control by paired t-tests.

important to note that there is always a possibility for some compounds to have appeared in more than one solvent, due to incomplete extraction.

Among the four different karela extracts, only hexane and water extracts were found to be active in improving oral glucose tolerance in our NIDD model rats. This suggests that Thai karela contains at least two active components: a non-polar compound and a very polar compound. The slight improvement (though not significant) on oral glucose tolerance by the methanol extract (only after cumulative treatment) may be due to the presence of traces of the very polar component which was predominantly present in the water extract.

Our results generally supported the findings by previous researchers. For example, Venkanna Babu *et al.* (1988) reported that glucose tolerance in alloxan recovered rabbits was improved by oral administration of a benzene extract of karela, but not an ethanolic extract. In this instance, ethanol, with a similar polarity as methanol, might not be polar enough to extract the very polar active component(s). In addition, Higashino *et al.* (1992) reported that a polar solvent extract of karela improved tolerance of orally administered glucose.

One possible identity of the active component present in the water extract might be steroidal glycosides, such as charantin, a mixture of sitosterol and stigmastadienol glucosides, which had been previously reported as the active compound by Lotlikar and Rajarama Rao (1966). If this is the case, then the aglycone of the glycoside might be extracted in the hexane extract which is responsible for the activity. This is further investigated in a latter chapter (Section 5.8).

In addition, it is interesting to look at the dosages of hexane and water extracts which gave a significant improvement in oral glucose tolerance. About 2mg/kg of hexane extract was enough to elicit a response, whereas about 100mg/kg of water extract was required for a similar response. Though the improvement of oral glucose tolerance by both extracts was much less than that produced by metformin, a much higher dose of metformin was employed in the tests (200mg/kg).

The possible stimulation of phase I insulin release by both hexane and water extracts corresponds to the insulin stimulation by whole karela juice reported earlier (Section

3.2.2(b)) although this was not observed in Section 3.3. These results again suggested that there may be a connection between the anti-hyperglycaemic effect of karela and the stimulation of phase I insulin release. This is based on the fact that only the hexane and water extracts which exhibited an initial rise in plasma insulin resulted in a significant improvement in oral glucose tolerance, whereas both chloroform and methanol extracts did not stimulate phase I insulin release and also had no significant effect on oral glucose tolerance.

3.6 Investigation of the mode of action of karela extracts

The previous experiment illustrated that both hexane and water extracts had significant effect on oral glucose tolerance. In order to further understand the mode of action of these extracts, their effects on intravenous and intraperitoneal glucose tolerance were studied.

3.6.1 Effects on intravenous glucose tolerance test (IVGTT)

Method

On Control Day, either water (10ml/kg) or 0.3% v/v Tween 80 (10ml/kg) was orally administered to 2h fasted rats, i.e. 45 min before an intravenous glucose (0.5g/kg) challenge. The rats were then immediately anaesthetised by intraperitoneal injection of sodium pentobarbitone (Sanofi; 5mg/100g body weight (see Appendix B)). Blood samples (0.3ml) were drawn from the tail tip at 0 min (just before glucose was administered intravenously via the saphenous vein) and at 5, 10, 15, 20 and 30 min after glucose administration. The rats were unconscious throughout this period.

Three days later, 5 groups of rats (n=6) were orally dosed with one of the following for 4 consecutive days: either water (10ml/kg) or 0.3% v/v Tween 80 (10ml/kg) as negative controls, metformin (200mg/kg; 200mg in 10ml) as a positive control, or hexane extract (10ml/kg) or water extract (10ml/kg) as test substances. The dosages of extracts used in the testing were the same as in OGTT (Section 3.5.1). On Test Day 4, (as on Control Day), the rats were anaesthetised and intravenous glucose was given 45 min after the test substances and blood sampling was carried out at the same time points. Plasma glucose and insulin were measured.

Results

The experimental model was validated with the results obtained with the negative controls (water and 0.3% v/v Tween 80) and the positive control (metformin). From the results shown in Fig. 3.10, both extracts, after cumulative administration, did not improve intravenous glucose tolerance, and neither did they have any stimulatory effects on plasma insulin (Table 3.11). In fact, the water extract had a tendency of reducing plasma insulin level, in which the level at two of the time points reached statistical significance.

3.6.2 Effects on intraperitoneal glucose tolerance test (IPGTT)

Method

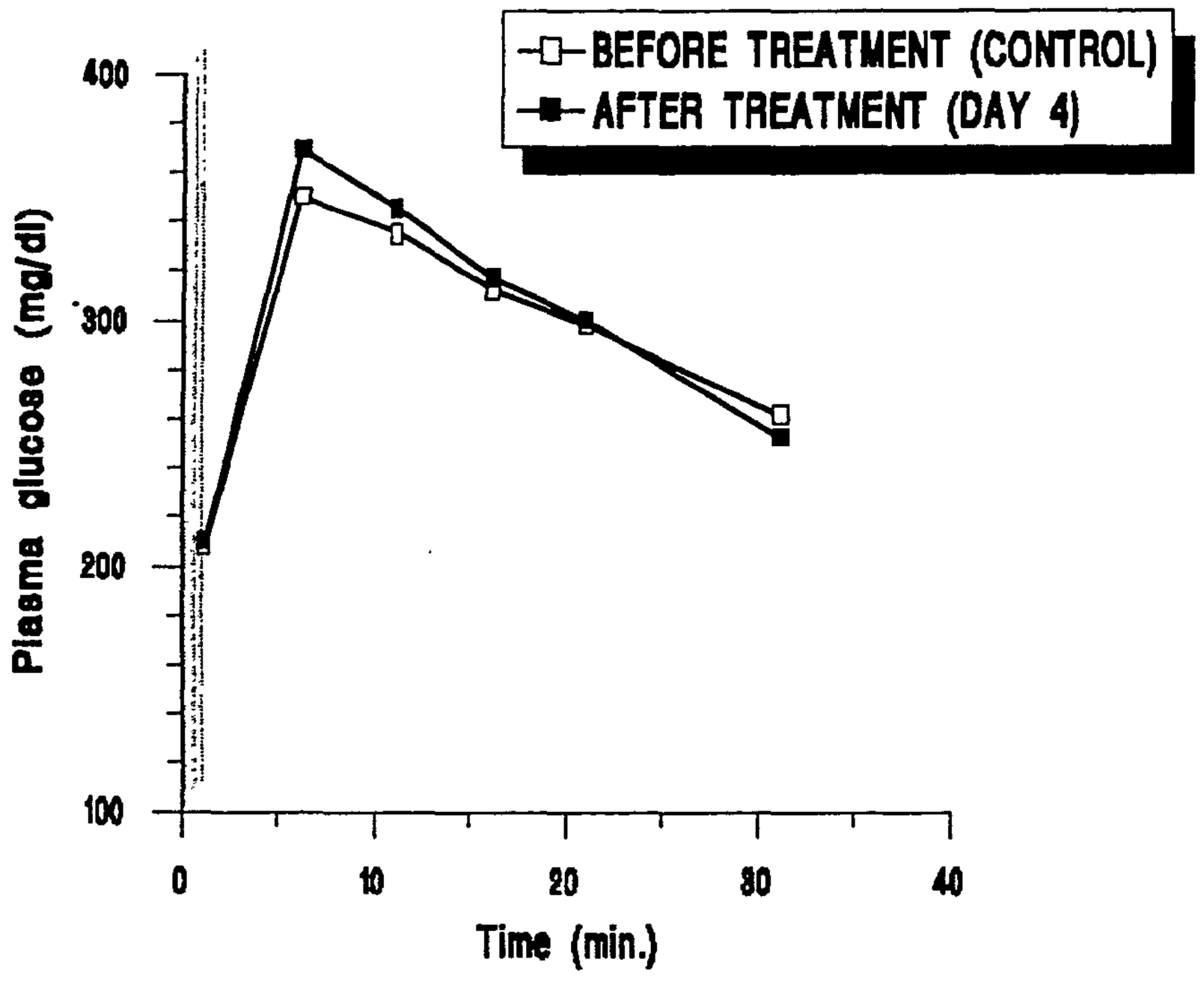
On Control Day, either water (10ml/kg) or 0.3% v/v Tween 80 (10ml/kg) was orally administered to 2h fasted rats, 30 min before an intraperitoneal (using a needle) glucose (2g/kg; 2g in 5ml) challenge. Blood samples (0.3ml) were drawn from the tail tip at 0 min (just before glucose was administered intraperitoneally) and at 5, 10, 15, 20, 30, 45, 60 and 90 min after glucose administration. The rats were kept unfed throughout this period and no anaesthetic was required.

Three days later, 5 groups of rats (n=6) were orally dosed with one of the following for 4 consecutive days: either water (10ml/kg) or 0.3% v/v Tween 80 (10ml/kg) as negative controls, metformin (200mg/kg; 200mg in 10ml) as a positive control, hexane extract (10ml/kg) or water extract (10ml/kg) as test substances. The dosages of extracts used in the experiment were the same as in OGTT (Section 3.5.1). On Test Day 4, as on Control Day, intraperitoneal glucose was given 30 min after the test substances and blood sampling was carried out at the same time points. Plasma glucose and insulin were measured.

Results

The experimental model was validated with the results obtained with the negative controls (water and 0.3% v/v Tween 80) and the positive control (metformin). From the results (Fig. 3.11), hexane extract, but not water extract, improved intraperitoneal glucose tolerance after 4 days of treatment. The plasma glucose at most time points were significantly reduced. As shown on Fig. 3.12, the hexane extract appeared to show some stimulation of insulin release compared to control at 10 and 15 min time points,

a)



b)

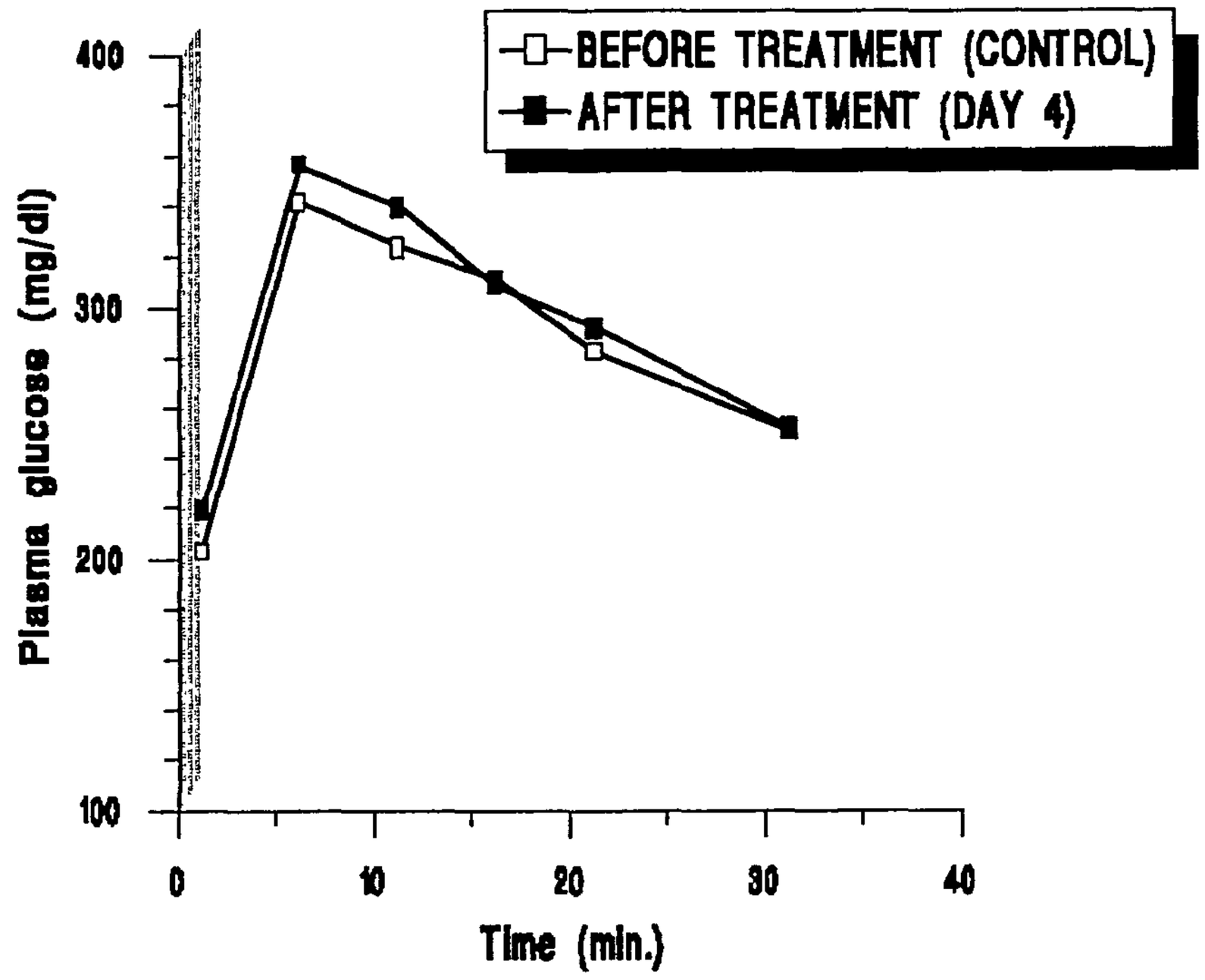


Figure 3.10: Effect of orally administered *a) Hexane extract* (10ml(2.2mg)/kg body weight) and *b) Water extract* (10ml(102.7mg)/kg body weight) on intravenous glucose tolerance (0.5g/kg) in 2h fasted n0 STZ diabetic rats (n=6) after four days of treatment. Glucose challenge was given at time 0; karela extracts were given 45 min. before glucose.

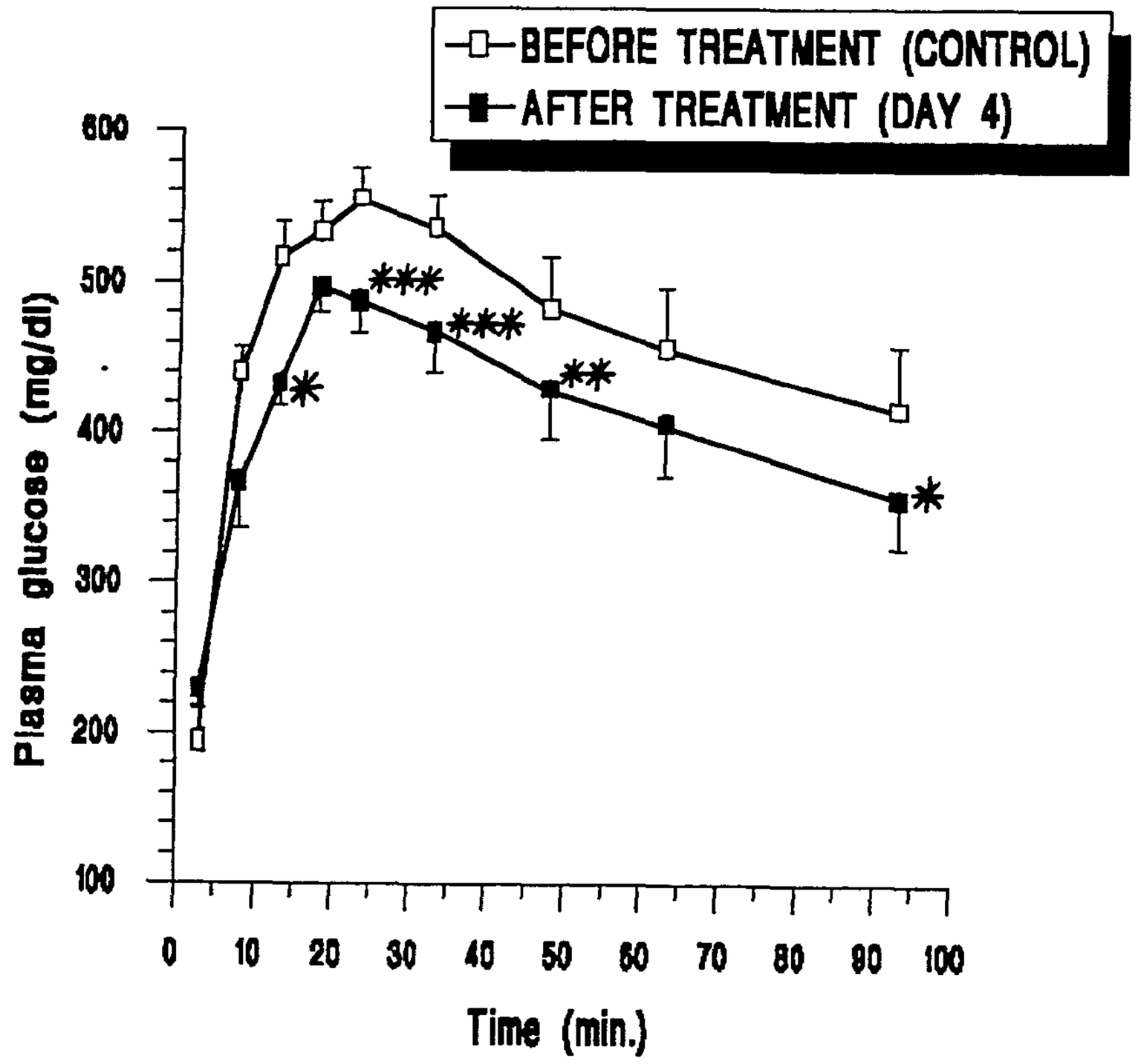
N.B. For the sake of clarity, vertical bars representing standard error of mean are not shown.

Table 3.11: Effect of orally administered hexane extract (10ml(2.2mg)/kg body weight) and water extract (10ml(102.7mg)/kg body weight) on plasma insulin in response to an intravenous glucose load (0.5g/kg) in 2h fasted n0 STZ diabetic rats (n=6) after four days of treatment. Glucose challenge was given at time 0; karela extracts were given 45 min. before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)					
	0 min	5 min	10 min	15 min	20 min	30 min
Hexane extract						
Control Day (n=6)	40.0 ± 4.6	57.8 ± 3.4	62.7 ± 6.2	63.5 ± 5.6	68.5 ± 5.8	79.0 ± 7.6
Day 4 (n=6)	42.7 ± 2.9	62.8 ± 6.6	57.8 ± 6.0	68.8 ± 5.3	70.0 ± 6.4	76.5 ± 6.6
Water extract						
Control Day (n=6)	62.0 ± 7.8	71.8 ± 7.7	81.5 ± 8.5	85.8 ± 8.3	84.0 ± 7.9	88.2 ± 10.1
Day 4 (n=6)	43.0 ± 2.7*	61.7 ± 6.2	66.7 ± 6.4*	70.3 ± 7.1	85.8 ± 17.1	81.5 ± 9.6

a)



b)

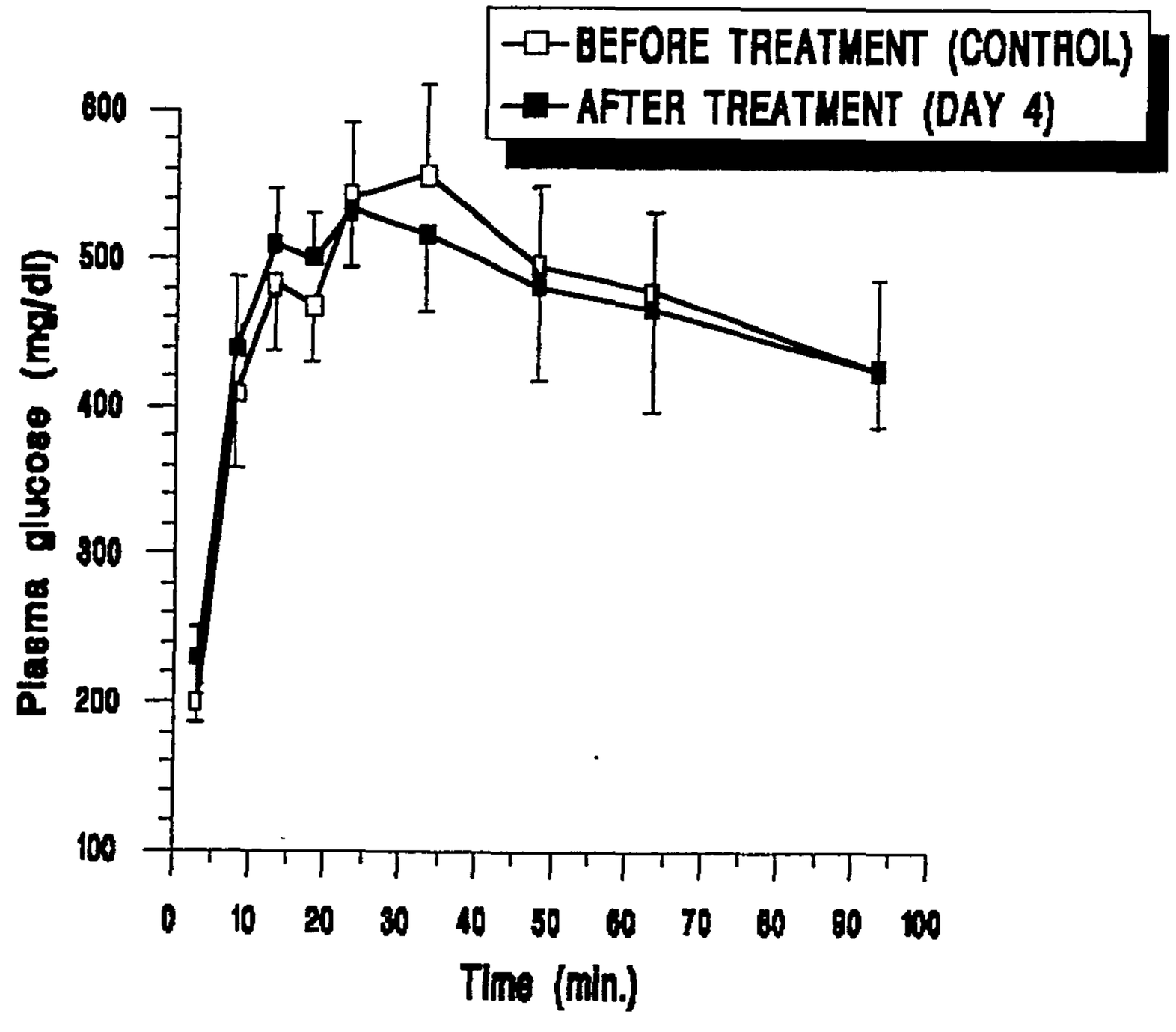
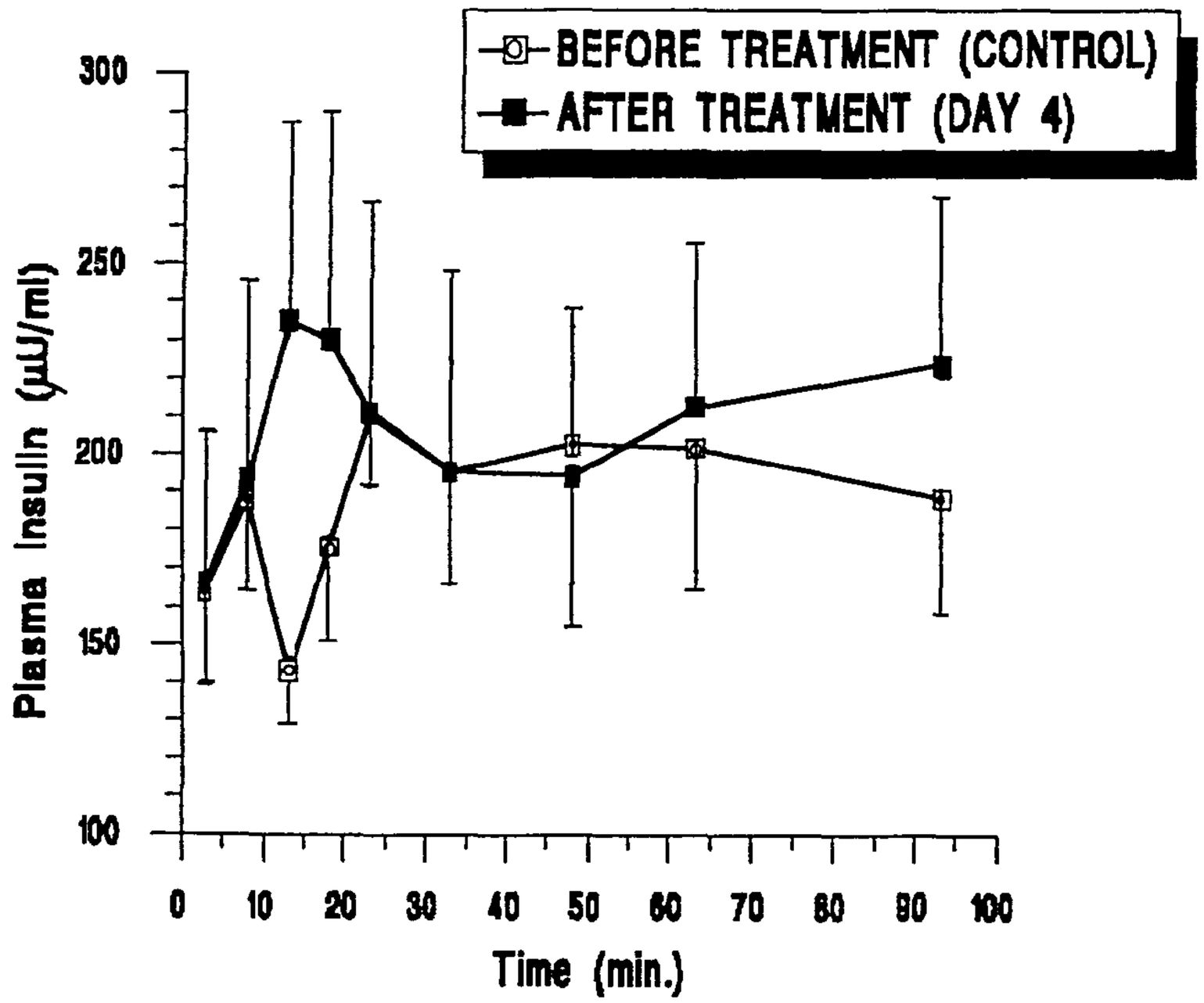


Figure 3.11: Effect of orally administered *a) Hexane extract* (10ml(2.2mg)/kg body weight) and *b) Water extract* (10ml(102.7mg)/kg body weight) on intraperitoneal glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min. before glucose. Values of plasma glucose are mean +/- SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control, by Student's paired t-tests

a)



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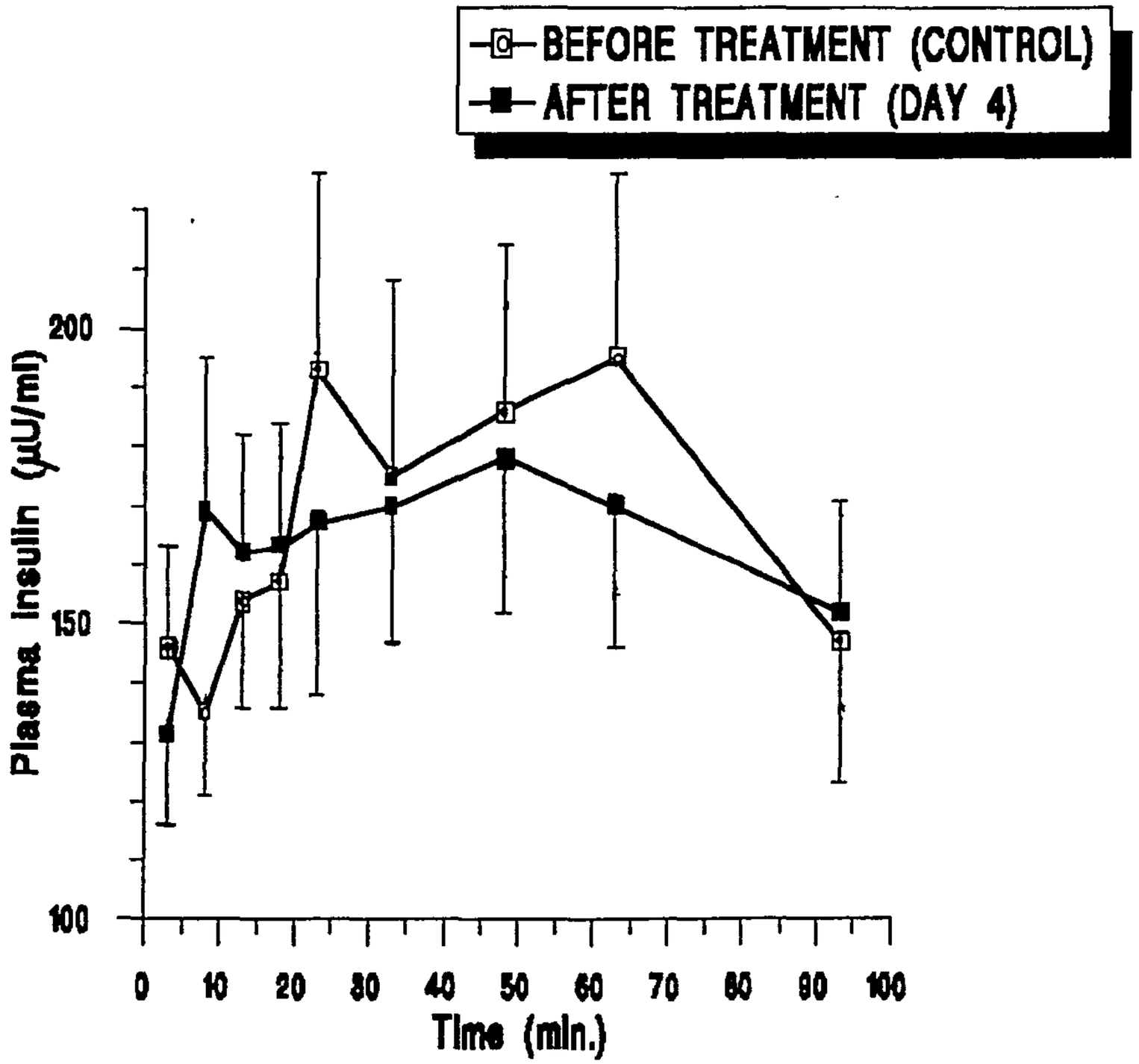


Figure 3.12: Effect of orally administered *a) Hexane extract* (10ml(2.2mg)/kg body weight) and *b) Water extract* (10ml(102.7mg)/kg body weight) on plasma insulin in response to an intraperitoneal glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min. before glucose. Values of plasma insulin are mean +/- SEM.

though this was not statistically significant (p = 0.081 and 0.282 respectively). The water extract also resulted in an initial rise in mean plasma insulin compared to control (at 5 min time point), which again did not reach statistical significance (p = 0.188).

3.6.3 Discussion

In the previous section (Section 3.5), both hexane and water extracts of karela juice were shown to improve oral glucose tolerance. By further studying their effects on intravenous and intraperitoneal glucose tolerance, one would expect a clearer picture on the mode of action of the two extracts to be revealed. From the results obtained, both hexane and water extracts significantly improved oral glucose tolerance but not intravenous glucose tolerance implying that Thai karela may inhibit glucose absorption from the gastrointestinal tract. Our result supports the finding by previous researchers (Meir and Yaniv, 1985) where *in vitro* glucose uptake by inverted gut was shown to be inhibited in the presence of extracts of karela fruit. However, no previous data could be found on the effect of karela juice/extracts on intravenous glucose tolerance. To the best of our knowledge, this is possibly the first IVGTT study carried out on karela.

It is also interesting to note that only hexane extract and not water extract improved intraperitoneal glucose tolerance. This is contradictory to the results reported by Day *et al.* (1990) and Higashino *et al.* (1992) where in both cases, a polar solvent extract (aqueous extract used by Day *et al.*) of karela was found to improve tolerance of both orally and intraperitoneally administered glucose. Regarding the mode of action, our results suggest that the hexane extract may also have some effects on the liver, since it seems to work when glucose passes through the hepatic portal vein (oral and intraperitoneal administration), whereas the water extract works primarily on the gut. In fact, the hepatic insulinomimetic effect of karela have been reported in previous studies. For example, in a recent study by Shibib *et al.* (1993), liver glucose-6-phosphate dehydrogenase (G-6-PDH) activity was elevated on *in vivo* administration of karela ethanolic extract by gastric intubation. This would enhance the utilisation of glucose by the liver leading to a lowering in blood glucose. In addition, the karela ethanolic extract was also reported to cause a depression in gluconeogenic enzymes

(glucose-6-phosphatase and fructose-1,6-biphosphatase) which may again account for the hypoglycaemic effect. However, it is unlikely that the active compound(s) present in the polar ethanolic extract would also be present in our non-polar hexane extract.

Thus from the results obtained so far, it is very likely that karela exhibits its anti-hyperglycaemic activity via a combination of modes of action.

3.7 Conclusion

In the following chapters, research work will be focused entirely on the hexane extract of karela (since this was the most active extract among those tested), in which its mode of action and its phytochemistry will be further investigated.

In order to further investigate the effect of karela on glucose absorption in the gut, an *in vitro* model of intestinal glucose absorption using brush border membrane vesicles was employed. Detailed experimental procedures regarding the use of the *in vitro* model and the results obtained will be discussed in the next chapter (see Chapter 4).

Chapter 4:

<u>In vitro</u> studies on karela fruit

Chapter 4: In vitro studies on karela fruit

4.1 Introduction

4.1.1 Glucose transport

Glucose is the major metabolic fuel for all mammalian cells. Glucose is transported across the small intestine by a two stage process: firstly, the entry of glucose into the epithelium across the brush border membrane; secondly, the efflux of glucose out of the cell across the basolateral membrane into the extracellular fluid near the blood capillaries, ready for absorption. Distinctly different glucose transport systems have been shown to be present in the two types of plasma membranes isolated from the small intestine (Murer *et al.*, 1974). The major transport mechanism in the brush border membrane is the Na^+/glucose co-transport system, SGLT1, where glucose is transported across the membrane into the cells against its concentration gradient through a coupling to the sodium electrochemical potential gradient across the membrane (Semenza *et al.*, 1984; Hediger *et al.*, 1987). A low intracellular concentration of Na^+ is maintained by the Na^+/K^+-ATPase localised in the basolateral membrane (Stirling, 1972). Phlorizin (Fig. 1.9) binds specifically to the Na^+/glucose transporter, inhibiting glucose uptake from the gut (Sigrist-Nelson and Hopfer, 1974). The glucose accumulated into the intestinal epithelial cells is then transported across the basolateral membrane by a Na^+-independent facilitated diffusion system (facilitative glucose transporter, GLUT-2) located on the basolateral membrane (Wright *et al.*, 1980; Thorens, 1992; Mueckler and Holman, 1995).

4.1.2 Fructose transport

Sigrist-Nelson and Hopfer (1974) had suggested the existence of a distinct Na^+-independent transport system in the brush border membrane for D-fructose, which is different from that of D-glucose. The fructose transporter was later found to be facilitative transporter GLUT-5 (Mahraoui *et al.*, 1992; Hyun and Martello, 1995).

4.1.3 Use of an *in vitro* brush border membrane vesicle model

As discussed in Section 1.2, for a compound to be anti-diabetic, one of the modes of action is to inhibit glucose absorption from the gastrointestinal tract. In the last chapter, both hexane and water extracts of karela were shown to improve oral

glucose tolerance (Fig. 3.8) but not intravenous glucose tolerance (Fig. 3.10) in n0 STZ NIDDM model rats, suggesting that they may in fact work by inhibiting glucose uptake from the gut. In order to investigate this particular mode of action, an *in vitro* model using intestinal brush border membrane vesicles was employed.

4.2 Brush border membrane vesicles (BBMV)

4.2.1 Introduction

Brush border membrane vesicles are made from the tips of the microvilli of small intestine. BBMV are enclosed in a double-layer unit membrane, the interior representing the serosal side. They are spherical, with an average diameter of 350nm, as shown in Fig. 4.1 (Wood, 1991). The vesicles are tightly sealed (Semenza *et al.*, 1984), thus they can accumulate solutes against a concentration gradient. More than 90% of vesicles have the correct orientation to allow solute flux *in vitro* which correctly models that *in vivo* (Haase *et al.*, 1978).

Advantages of the BBMV absorption model:

a) BBMV are a microvillar membrane preparation free of organelles and endogenous substrates, and virtually free of metabolising enzymes. They enable the membrane events associated with absorption to be examined in isolation, in the absence of any metabolic effects (Kessler *et al.*, 1978).
b) BBMV are one of the most stable intestinal preparations available for studying transport.
c) BBMV require only small amount of substrates (µl volumes).
d) BBMV are a very well-characterised model in terms of glucose uptake (Hopfer *et al.*, 1973; Semenza *et al.*, 1984; Wood, 1991).

Here, BBMV were used as an *in vitro* model for studying the effect of karela juice/extracts on glucose uptake.

4.2.2 Materials and method

Brush border membrane vesicles were made from the tips of the microvilli of frozen rabbit small intestine. Frozen rabbit intestines were prepared as in Fig. 4.2. BBMV were prepared using the Mg^{2+} precipitation method proposed by Schmitz *et al.* (1973), with the modifications of Kessler *et al.* (1978), summarised in Fig. 4.3. All

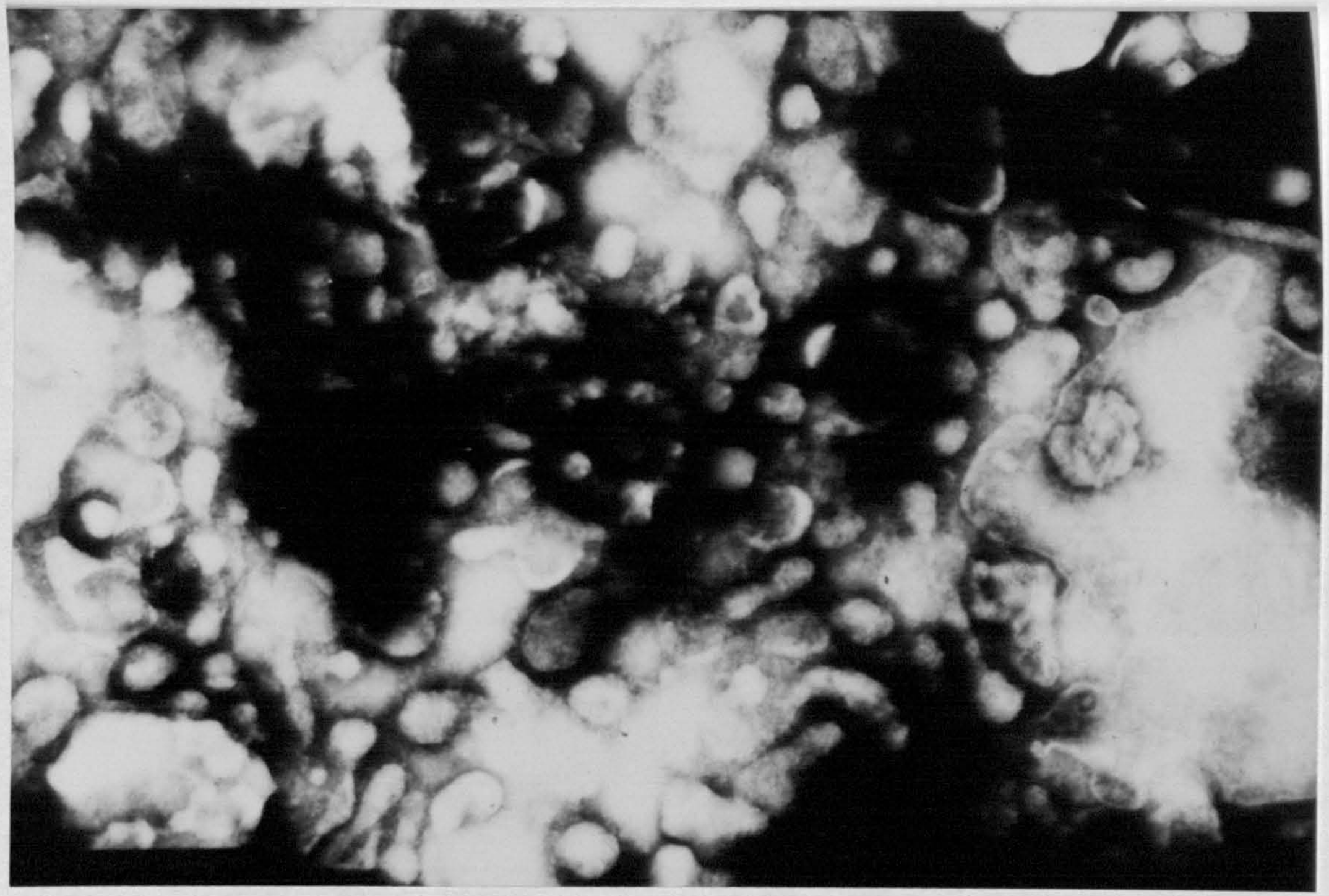


Figure 4.1: A representative transmission electron micrograph of BBMV (Magnification ×66000)

(Photo reproduced from the PhD thesis of Wood (1991))

A Formvar coated 400 mesh copper grid strengthened with carbon was laid on top of a drop of BBMV suspension for 90 seconds. The wet grid was transferred to 0.2% ammonium molybdate (pH 5.0) for 2 minutes. Excess stain was removed with a wick of filter paper. Vesicle size was calculated from the micrograph by measuring vesicle diameter.

Freshly killed white New Zealand rabbit
(by intravenous injection of pentobarbitone, 60mg/kg body weight)

↓

Small intestine was dissected from the pyloric sphincter to ileo-caecal junction, and the mesentery was separated from the intestine

↓

Small intestine was placed in 200ml ice-cold 154mM KCl

↓

The remaining mesentery and fat were removed, contents of the intestine were gently squeezed out

↓

The small intestine was cleaned in 200ml fresh ice-cold KCl

↓

Small intestine was cut longitudinally, washed thoroughly in KCl, and blotted dry between tissues

↓

Small intestine was weighed and snap frozen in a plastic bag in liquid nitrogen

↓

Frozen small intestine was stored at -70^0C

Figure 4.2: Flow chart for the preparation of frozen rabbit small intestine

Frozen small intestine was thawed in 200ml Buffer 1 and was cut into small pieces

↓

Mechanically vibrated for 1 minute using a Vibro-mixer (Vibromax)

↓

Solution was filtered under vacuum and the supernatant was made up to 300ml with Buffer 1

(Connective tissues and muscles were removed)

↓

0.61g $MgCl_2$ was added and stirred

(Magnesium precipitation of nuclei, mitochondria, most of basolateral membranes and other cell debris)

↓

Solution was centrifuged at 4^0C, 5000rpm, for 15 minutes

↓

The pellet was discarded, the supernatant was centrifuged at 4^0C, 17000rpm, for 30 minutes

↓

The supernatant was discarded, the pellet was resuspended in Buffer 2

↓

The solution was centrifuged at 4^0C, 17000rpm, for 40 minutes

↓

The supernatant was discarded, the pellet was resuspended in 1ml Buffer 3

↓

The pelleted volume was measured and it was doubled using Buffer 3

↓

The solution was passed 5 times through a 25 gauge needle

(BBMV vesicles were formed)

↓

The solution was divided into 0.26ml aliquots and snap frozen in liquid nitrogen

Buffer 1: 10mM mannitol, 2mM HEPES-Tris*, pH 7.1
Buffer 2: 100mM mannitol, 0.1mM $MgSO_4$, 2mM HEPES-Tris, pH 7.4
Buffer 3: 300mM mannitol, 10mM HEPES-Tris, 0.1mM $MgSO_4$, pH 7.4

* HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Tris = 2-amino-(hydroxymethyl)propane-1,3-diol

Figure 4.3: Flow chart for the preparation of BBMV

chemicals and reagents used were purchased from Sigma.

4.3 Studies on glucose uptake using brush border membrane vesicles

4.3.1 Materials and method

The uptake of D-glucose into BBMV was performed according to the rapid filtration technique of Hopfer *et al.*, 1973.

One aliquot (0.26ml) of frozen BBMV was resuspended in 0.44ml of Buffer 3 (300mM mannitol, 10mM HEPES-Tris, 0.1mM $MgSO_4$, pH 7.4). BBMV (20µl) were mixed with 40µl of 0.1mM D-glucose (Sigma) solution {0.1mM D-glucose in glucose incubation buffer (100mM NaSCN, 100mM mannitol, 10mM HEPES-Tris (Sigma), pH 7.4) containing D-[2-^{3}H]glucose (Amersham) to a specific activity approximately 25000cpm} and placed in a water bath at 25^{0}C. In order to obtain a time profile of glucose uptake into BBMV, glucose uptake was stopped after certain incubation times (0, 10, 20, 40, 60, 120, 300 and 3600 seconds) by addition of 1ml ice-cold stop-wash buffer (200mM NaCl, 10mM HEPES-Tris (pH 7.4), 250µM phlorizin (Fig. 1.9; Sigma)) to the incubated mixture, followed by rapid filtration through a pre-wetted 0.45µm cellulose acetate/nitrate Millipore filter. The filter was washed 5 times with 1ml of the same ice-cold stop-wash buffer. The filters, which retained the BBMV, were each placed in a minivial together with 3.5ml scintillation fluid (Ready-protein scintillation cocktail; Beckman-RHC Ltd.) and were counted for 5 minutes in the appropriate [^{3}H] channel of the liquid scintillation counter (LKB Wallac Instruments Ltd.). All reactions were performed with 3 repeats and the mean values were calculated. Control incubations containing 40µl of 0.1mM [^{3}H]-labelled D-glucose solution (without the addition of BBMV) were also filtered as above in order to measure non-specific binding of D-glucose to the filters, and corrections were applied to the glucose uptake values.

Glucose uptake was expressed as picomoles (pmoles, 10^{-12}) of glucose/mg protein using the following equation:

$$\text{Glucose uptake} = \frac{\text{corrected cpm}}{\text{mean cpm for control samples}} \times \frac{\text{amount of glucose added (pmoles)}}{\text{protein concentration of BBMV} \times \text{volume of BBMV (ml)}}$$

Key:

Corrected cpm (counts per minute) =

actual measured value – value for non-specific binding to filter.

Mean control cpm =

total activity (in this case, the activity of 40µl 0.1mM radiolabelled D-glucose).

Amount of glucose added in pmoles:

in this case, 40µl of 0.1mM D-glucose,

0.1mM ≡ 0.1mM/L ≡ 0.1µM /ml ≡ 0.1nM/µl ≡ 100pmoles/µl,

i.e. for 40µl, there were 4000pmoles.

volume of BBMV added: in this case, 20µl ≡ 0.02ml.

The protein concentration of BBMV suspension was determined according to the method of Bradford (1976), with modifications by Read and Northcote (1981). This protein determination method involved the binding of the dye, Coomassie Brilliant Blue G-250 ($C_{47}H_{48}N_3O_7S_2Na$), to the protein sample, followed by measurement of the change in absorbance at 595nm due to the formation of a blue dye-protein complex. Protein concentration was expressed in mg/ml.

Preparation of dye reagent

10mg of Coomassie Brilliant Blue G-250 (Sigma) was dissolved in 5ml of 95% ethanol. To this solution, 10ml of 85%w/v phosphoric acid (BDH chemicals Ltd.) was added. The resulting solution was diluted to a final volume of 100ml. The final concentrations in the reagent were 0.01%w/v Coomassie Brilliant Blue G-250, 4.7%w/v ethanol and 8.5%w/v phosphoric acid.

Preparation of BBMV test samples

One aliquot (0.26ml) of frozen BBMV was resuspended in 0.44ml of water (instead of Buffer 3). Three dilutions (using water) were made for the assay: 1 in 10; 1 in 50 and 1 in 100 dilutions.

Protein assay

Bovine serum albumin (Fraction V, 98-99% albumin; Sigma) was used. A standard protein solution 1mg/ml was prepared. The preparation of standard solutions for the calibration curve was shown in Table 4.1.

Table 4.1: Preparation of standard solutions for the calibration curve for protein assay

Solutions	Volume of standard (ml)	Volume of water (ml)	Protein concentration (mg/ml)
A	0.0	1.0	0.0
B	0.2	0.8	0.2
C	0.4	0.6	0.4
D	0.6	0.4	0.6
E	0.8	0.2	0.8
F	1.0	0.0	1.0

Assay procedure

For the calibration curve, dye reagent (950µl) was added to 50µl of protein solutions A-F containing 0-50µg of protein. Absorbance at 595nm (A_{595}) was measured (using a UV/VIS spectrophotometer; Perkin Elmer UV/VIS Spectrometer Lambda 2) against a water blank within the first hour, since the dye binding process completed in approximately 2 min with good colour stability for 1 hour (Bradford, 1976). All measured A_{595} were corrected by subtraction of the A_{595} obtained using solution A. Samples were assayed in duplicate and mean results were obtained. The same assay procedures were used for the BBMV test samples, i.e. 950µl of dye reagent was added to 50µl of each of the three dilutions of BBMV samples. Thus the protein concentration of BBMV can be calculated by taking the mean of the three results obtained from the 3 dilutions. The protein assay was carried out for each batch of BBMV.

4.3.2 Results

The results of the protein assay for a particular batch of BBMV are presented in

Appendix 5. In this case (Appendix 5), the protein concentration was 3.5mg/ml. Using the equation, mean glucose uptake (expressed as pmoles/mg protein) at each time point was calculated. A typical glucose uptake profile in BBMV is drawn and shown in Fig. 4.4. From the graph, a rapid initial glucose uptake was observed which reached the peak at 20 seconds (696.8 pmoles/mg protein), and then gradually declined to below 100 pmoles/mg protein at 3600s.

4.3.3 Discussion

According to Hopfer *et al.* (1973), glucose uptake by BBMV represents entry into an intravesicular aqueous space rather than binding to the membrane. In addition, the presence of sodium in the medium (but not in the intravesicular spaces) stimulates D-glucose transport. In our study, the presence of sodium thiocyanate in the glucose incubation buffer (outside BBMV) provides the Na^+ required for the co-transport of D-glucose. The glucose uptake profile showed a large overshoot which is transient since no ion pumps are present in BBMV as required to maintain the inwardly directed sodium gradient. Thus a transient accumulation of D-glucose was seen to occur initially, however, once the driving ion gradient was dissipated, the accumulated glucose declined by diffusion out of the vesicles towards equilibrium.

4.4 Effects of karela juice on glucose uptake using BBMV model

The effect of Thai karela juice on glucose uptake was studied using BBMV model.

4.4.1 Materials and method

Freshly prepared Thai karela juice was used in the study (method as described in Section 3.2.1).

One aliquot (0.26ml) of frozen BBMV was resuspended in 0.44ml of Buffer 3 (300mM mannitol, 10mM HEPES-Tris, 0.1mM $MgSO_4$, pH 7.4). For the control, 20µl BBMV was mixed with 40µl of 0.1mM D-glucose solution {0.1mM D-glucose in glucose incubation buffer (100mM NaSCN, 100mM mannitol, 10mM HEPES-Tris, pH 7.4) containing D-[2-^{3}H]glucose to a specific activity approximately 25000cpm} and placed in a water bath at 25^0C. For the test, 20µl BBMV was mixed

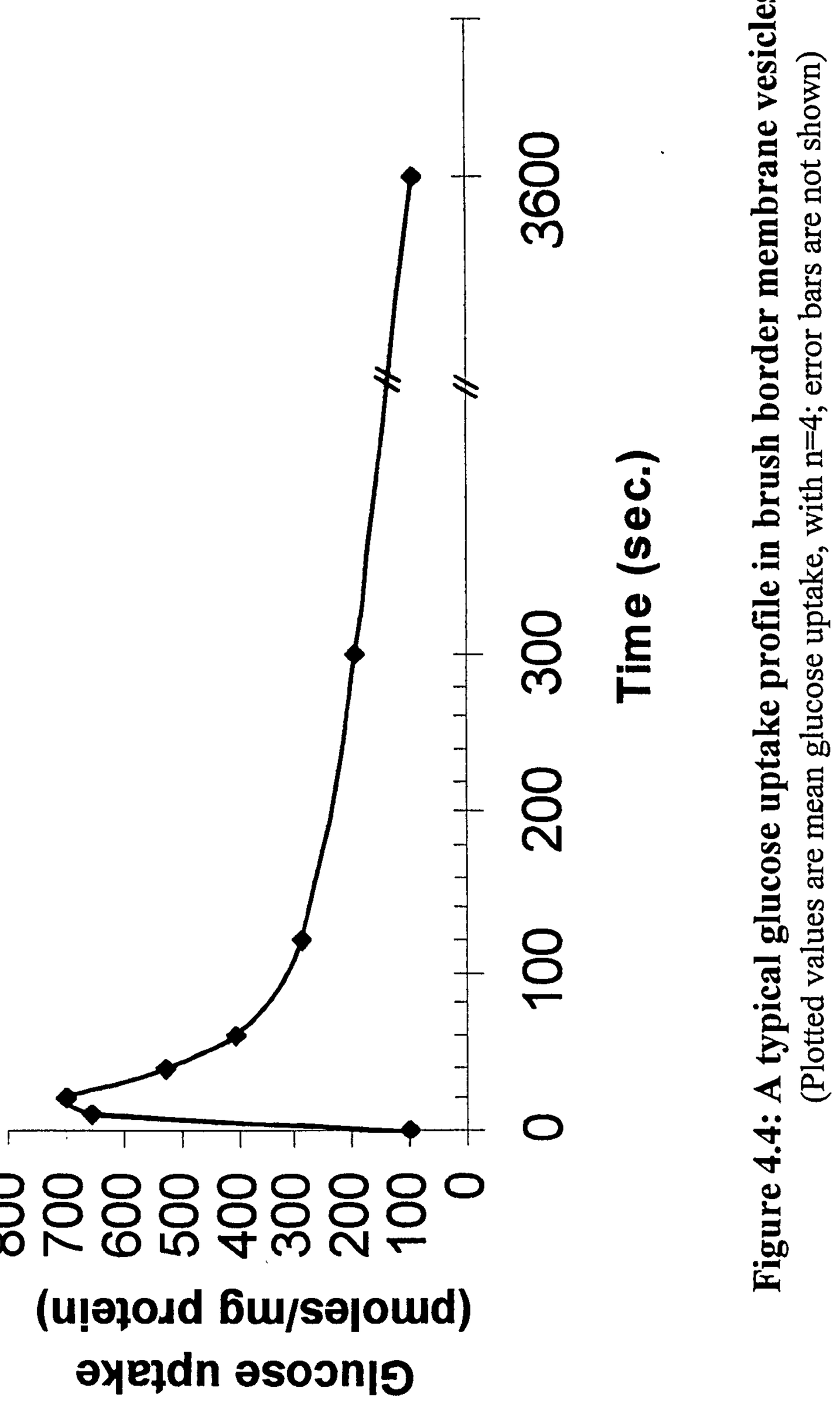


Figure 4.4: A typical glucose uptake profile in brush border membrane vesicles
(Plotted values are mean glucose uptake, with n=4; error bars are not shown)

with 20µl of 0.2mM D-glucose solution {0.2mM D-glucose in glucose incubation buffer of double concentration (200mM NaSCN, 200mM mannitol, 20mM HEPES-Tris, pH 7.4) containing D-[2-^{3}H]glucose} and 20µl karela juice (at different concentrations, see below). Glucose uptake was stopped after 20s incubation time (peak uptake, as shown in Fig. 4.4) by addition of 1ml ice-cold stop-wash buffer and the rest of the procedures were carried out as before (Section 4.3.1). All reactions were performed with 3 repeats and the mean values were calculated. Control incubations containing 20µl of 0.2mM [^{3}H]-labelled D-glucose solution and 20µl karela juice (at different concentrations), without the addition of BBMV, were used to measure non-specific binding of D-glucose to the filters. Glucose uptake was expressed as pmoles/mg protein as described before (Section 4.3.1). The test solutions used were: karela juice (neat), diluted karela juice --- 1 in 10, 1 in 100 and 1 in 1000 dilutions with water. In a separate experiment, instead of using water as the diluent, glucose solution (having the same concentration of glucose as in karela juice(10mM, Appendix 6)) was used to dilute karela juice to give 1 in 10, 1 in 100 and 1 in 1000 dilutions.

4.4.2 Results

4.4.2(a) Effect of karela juice (at different concentrations by dilutions with water) on glucose uptake

Results are shown in Fig. 4.5. As a control, the absence of karela juice resulted in no inhibition of glucose uptake into the vesicles. However, karela juice (neat) caused an approximately 91% inhibition of glucose uptake. As the juice was diluted (with water), the inhibitory effect was also reduced. Thus the more diluted the juice, the less inhibitory effect on glucose uptake.

4.4.2(b) Effect of karela juice (at different concentrations by dilutions with glucose solution) on glucose uptake

Results obtained are shown in Table 4.2. From the results, the dilution of karela juice (using glucose solution as diluent) did not affect the percentage of inhibition of glucose uptake into BBMV. All four different concentrations of karela juice gave very similar percentage of inhibition of glucose uptake into BBMV.

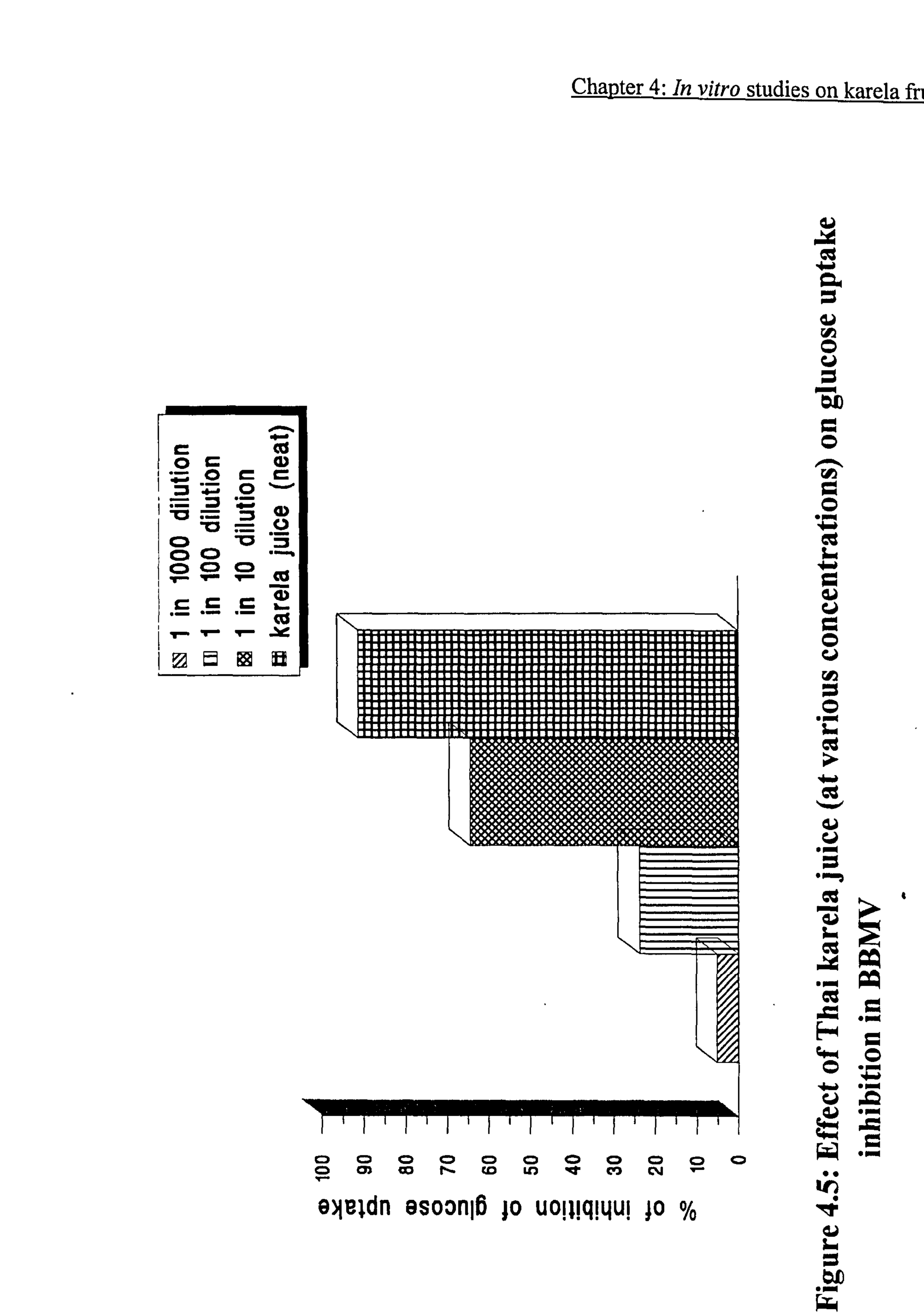


Figure 4.5: Effect of Thai karela juice (at various concentrations) on glucose uptake inhibition in BBMV

Table 4.2: Effect of various concentrations of karela juice (diluted with 10mM glucose solution) on glucose uptake into BBMV

Concentration of karela juice	% of inhibition of glucose uptake into BBMV
neat	92.0
1 in 10 dilution	90.1
1 in 100 dilution	91.3
1 in 1000 dilution	90.6

4.4.3 Discussion

The results (4.4.2(a)) obtained seem to confirm that karela juice inhibited glucose absorption from the gut. A clear dose-response effect was observed; the more diluted the juice, the less inhibition of glucose uptake into the vesicles. However, on closer examination of the experimental protocol, the inhibitory effect seen might not be totally related to karela juice. This is because karela juice contains D-glucose, as shown in the HPLC assay (Appendix 6). The analysis showed that Thai karela juice contained about 8-11mM glucose, which is much higher than the concentration of glucose used in this *in vitro* testing (0.1mM). Thus the glucose that is present in karela juice might have competed with the radiolabelled D-glucose and resulted in an apparent inhibitory effect of glucose uptake into the vesicles. In addition, the fact that D-fructose is present in the juice (Appendix 6) may also need to be considered. However, the results are unlikely to have been affected by the presence of D-fructose in the juice since the fructose transporter in the brush border membrane is known to be different from that for the transport of D-glucose (Section 4.1.2). In fact, other results obtained in our laboratory (Srijayanta *et al.*, 1997) have shown that fructose has no effect on glucose uptake by BBMV.

As a result, further investigations have to be carried out in order to confirm the results. To overcome this problem, one possible solution is to remove all glucose from karela juice by techniques such as dialysis or size exclusion chromatography, and then repeat the experiment, but this could prove to be time consuming. An alternative is to dilute karela juice in glucose solution (having same concentration of glucose as in the juice) instead of water. This would maintain the same glucose

concentration throughout the dilutions of karela juice, whilst any active compounds would be diluted, thus allowing a possible dose-response effect to be seen. The latter was carried out and from the results (Section 4.4.2(b)), the dilution of karela juice did not affect the percentage of inhibition of glucose uptake into BBMV. Thus we can confirm that the presence of D-glucose in karela juice (in a relatively high concentration (around 10mM) as compared to that of D-glucose used in this experiment (0.1mM)) competed with the radiolabelled D-glucose and resulted in an apparent inhibitory effect of glucose uptake into the vesicles. Thus the inhibitory effect of glucose uptake (if any) caused by the active components in karela juice would have been masked.

4.5 Effects of hexane extract of karela on glucose uptake using BBMV model

Hexane extract of karela was chosen for further study using the BBMV model because:

i) The positive result of hexane extract on OGTT and its lack of effect on IVGTT suggests that it may work by inhibiting glucose absorption from intestine.

ii) Hexane, a non-polar solvent, is not expected to extract any glucose present in karela juice. Thus the hexane extract should have the advantage of containing no glucose (confirmed by the HPLC assay (Appendix 6)), as opposed to whole karela juice, methanol and water extracts of karela which contain glucose.

4.5.1 Materials and method

Preparation of hexane extract

Hexane extract of karela was prepared and reconstituted in 0.3% v/v Tween 80 in water prior to testing as described before (Section 3.5.1).

In vitro testing using BBMV model

The same experimental procedures were used as above (Section 4.4.1). In this case, for the control, 20µl BBMV was mixed with 20µl of 0.2mM D-glucose solution {0.2mM D-glucose in glucose incubation buffer of double concentration (200mM NaSCN, 200mM mannitol, 20mM HEPES-Tris, pH 7.4) containing D-[2-

^{3}H]glucose} and either 20µl of water or 20µl of 0.3% v/v Tween 80. Here, water is used as an extra control to check if Tween 80 (the solubilising agent for redissolving hexane extract) itself had any inhibitory effect on glucose uptake into BBMV. For the test, 20µl BBMV was mixed with 20µl of 0.2mM radiolabelled D-glucose solution and 20µl of hexane extract of karela juice (redissolved in 0.3% v/v Tween 80). Glucose uptake was measured at 20s (peak uptake) and 3600s (equilibrium point).

4.5.2 Results

The results of the glucose uptake studies with hexane extract are shown in Table 4.3. From the results, 0.3% v/v Tween 80 had no significant effect on glucose uptake in BBMV when compared to control (water). However, the hexane extract at concentrations of 0.073µg/µl and 0.73µg/µl resulted in percentage inhibitions of glucose uptake of 10.8 ± 1.6% ($p = 0.075$, compared to control (0.3% v/v Tween 80) by unpaired t-test) and 33.5 ± 2.1% ($p < 0.0001$) respectively. There was no significant change in equilibrium glucose uptake at 3600s between Tween 80 and hexane extract at 10 times concentration, indicating that the BBMV probably remained intact.

4.5.3 Discussion

Our *in vitro* results supported the possibility that the hexane extract of karela inhibits intestinal glucose uptake. This explains the fact that hexane extract significantly improved oral but not intravenous glucose tolerance as we observed earlier in Chapter 3. However, since hexane extract was shown to improve intraperitoneal glucose tolerance (Section 3.6.2) as well, it may also have some effects on the liver (as already discussed in Section 3.6.3), in addition to the inhibitory effect on intestinal glucose absorption.

Our results also supported the previous findings by Meir and Yaniv (1985) in which glucose uptake by inverted gut was inhibited by acetone extract of karela. However, on closer examination of the experimental procedures, the author would question the accuracy of the reported inhibitory effect. This is because acetone extract is likely to contain glucose which is extracted from karela (our HPLC assay has shown the presence of glucose in karela, see Appendix 6) and the presence of unlabelled

Table 4.3: Effect of hexane extract of karela on glucose uptake into BBMV

Test substance	Glucose uptake at 20s (pmoles/mg protein) (Mean ± SEM, n=6)	Glucose uptake at 3600s (pmoles/mg protein) (Mean ± SEM, n=6)
Water (control)	425.1 ± 12.2	36.7 ± 8.9
0.3% v/v Tween 80 (control)	428.5 ± 20.3	30.5 ± 19.7
Hexane extract (at original concentration♦: 0.073µg/µl)	382.4 ± 6.8*	NP
Hexane extract (at 10× original concentration : 0.73µg/µl)	285.0 ± 9.1**	49.5 ± 6.7

N.B. i) *p < 0.1; **p < 0.0001 as compared to control (0.3% v/v Tween 80) by unpaired t-tests

ii) ♦ original concentration means that the residue of hexane extract was made up to the equivalent volume of karela juice from which it was extracted

iii) NP = not performed

glucose might have competed with radiolabelled [^{14}C] glucose used in the experiment and thus the observed inhibitory effect remains questionable.

The present study suggested that hexane extract may contain active compounds which act like phlorizin that inhibits Na^+-dependent/glucose transport in the brush border membrane. Although the IC_{50} (median inhibitory concentration) value of the hexane extract was not determined, by extrapolation, it would possibly be in the region of 1-5 μg/μl, i.e. 1-5 mg/ml. According to Johansen *et al.* (1998), phlorizin has an IC_{50} value of 0.1mM, i.e. about 0.05mg/ml in this model. Thus phlorizin may be 20-100 fold stronger than the hexane extract of karela as a Na^+-dependent glucose transport inhibitor.

Na^+-dependent glucose co-transporters are found in the small intestine as well as in the proximal tubule in the kidneys. If hexane extract contains phlorizin-like compound(s), the anti-hyperglycaemic effect may be a result not only of the inhibition of glucose uptake by the intestine, but also due to its inhibitory effect on the reabsorption of glucose by the kidneys resulting in glycosuria. To the best of our knowledge, the latter has never been fully examined. However, the absence of lowering effect of the hexane extract on intravenous glucose tolerance may indicate an absence of any effect on the kidneys.

4.6 Conclusion

Despite the fact that BBMV have been well-characterised in terms of glucose uptake, the author believes that this is the first time where BBMV were used as an *in vitro* model for studying natural products with intestinal glucose uptake inhibitory effect. In fact, with the advantages of low experimental cost and simple procedures, as well as the beauty of requiring only small amount of substrates for testing, BBMV are a good *in vitro* model for screening anti-diabetic plants for potential inhibitory effects on glucose uptake from the intestine.

The only drawback of the BBMV model is that the substrates used for testing should ideally be glucose free in order to have valid results. Thus in our present study, only the hexane, but not water, extract was tested since the water extract is likely to contain glucose which is present in karela fruit. Sugars can be removed from the

extract by different techniques (Houghton and Raman, 1998). One such method is size exclusion chromatography using standard hydrophilic exclusion gels such as Sephadex®. Here, sugars, being small molecules, are retained by the column and are eluted last, whereas the larger molecules in the extract pass through and are eluted first. In this way, sugars can be separated from other components in the extract.

Chapter 5:

Bioassay-guided fractionation of karela juice

Chapter 5: Bioassay-guided fractionation of karela juice

5.1 Introduction

The investigations described in Chapter 3 had revealed that both hexane and water extracts of Thai karela improved oral glucose tolerance in n0 STZ NIDDM rats, with the hexane extract being more potent. In *in vitro* experiments, we observed an inhibitory effect of hexane extract on glucose uptake in a BBMV model (Chapter 4). In this chapter, further studies carried out on the hexane extract with respect to isolating its active components will be described. Here, bioassay-guided fractionation using the *in vivo* n0 STZ NIDDM model was employed. The whole hexane extract was further fractionated by column chromatography, the sub-fractions obtained were used in oral glucose tolerance tests using the n0 STZ model. The active sub-fractions were further fractionated by column chromatography or preparative thin layer chromatography (PTLC) until pure compounds were obtained, whereas the inactive fractions were not studied further. The structures of the isolated compounds were elucidated using nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and gas chromatography-mass spectrometry (GC-MS).

All the NMR, MS and GC-MS spectra were run by the ULIRS NMR spectroscopy service, ULIRS mass spectrometry service and ULIRS GC-MS unit respectively in the Chemistry Department of King's College London.

^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded on a Bruker AMX-400 (400MHz) instrument. Additionally, two-dimentional Correlation Spectroscopy (COSY), Nuclear Overhauser Enhancement Spectroscopy (NOESY) and ^{13}C-^{1}H one bond correlation spectroscopy were obtained, wherever possible, for reference. Deuterated solvent, $CDCl_3$ was used in all cases, with Tetramethylsilane (TMS) as internal reference. Chemical shifts are expressed as δ values (ppm) downfield from TMS.

Electron Impact Mass Spectrometry (EIMS) measurements were recorded on high resolution instrument with resolution of 3500 using processing software DS90 against Kratos MS 890 instrument. A direct insertion probe was used for

measurement of the sample.

High resolution Fast Atom Bombardment (FAB) MS were recorded on a VG ZAB-SE using a xenon FAB gun. The sample was introduced by a matrix (nitrobenzylalcohol + sodium chloride) on a probe. Accurate mass measurements were recorded using peak matching at a resolution of 8-10000.

5.2 Fractionation of hexane extract by column chromatography

5.2.1 Materials and method

Solvents

Hexane (C_6H_{14}), methanol (MeOH), acetonitrile (CH_3CN) and chloroform ($CHCl_3$) were obtained from BDH Chemicals, UK.

Preparation of hexane extract

Fresh Thai karela fruit (15kg, Batch M) was bought and karela juice was prepared as described in Section 3.2.1. Karela juice (7.8L) was immediately freeze-dried into powder (232.15g). The freeze-dried powder was then extracted (using Soxhlet extraction) with hexane for 18 hours. Hexane extract was evaporated to dryness using a rotary vacuum evaporator and 2.2302g of residue was produced.

Column chromatography

Approximately 100g reverse-phase silica powder (LiChroprep® RP-18, particle size 40-63μm, Merck) was used to pack a column (30cm in length × 26mm internal diameter, with space in column of 28ml). The adsorbent was first made into a slurry with MeOH and loaded on the column, then pressure was applied and the adsorbent was compressed to a hard layer. Flash chromatography was used whereby the flow of solvent is increased by a positive pressure applied to the column, in this case, nitrogen gas was used. The dried hexane extract M2 (2.001g), premixed with minimum amount of hexane solvent, was applied to the top of the column of adsorbent. The column was then eluted by gradient elution of decreasing polarity, starting with MeOH, then MeOH: CH_3CN (2:1), followed by CH_3CN, $CHCl_3$ and finally C_6H_{14}.

N.B. This procedure is not suitable for further work since a non-polar solvent should not be present at the start of reverse-phase chromatography. It is recommended that the hexane solution is mixed with stationary phase and the solvent (hexane) removed by evaporation before the adsorbed sample is applied to the top of the column.

Thin layer chromatography (TLC)

Commercial pre-coated reverse-phase (RP-18 F_{254S}, Merck) plates were used for preliminary screening of crude extracts and to monitor fractions of extracts obtained from the column chromatography. In this case, the solvent system used was MeOH: CH_3CN (2:1).

5.2.2 Results

Figure 5.1 presents the schematic diagram for the extraction process of Thai karela fruit. 94 × 5ml fractions were initially collected from the column chromatography. Fractions with similar constituents were pooled together and eventually eleven sub-fractions (M2A-M2K) were obtained (Fig. 5.1). From the TLC zone profile (Fig. 5.2), each sub-fraction contains at least 2 components.

5.3 Effect of sub-fractions of hexane extract on oral glucose tolerance in n0 STZ model

In the previous section (5.2), the crude hexane extract was fractionated into 11 sub-fractions. Ideally, all these sub-fractions should be tested for their effects on oral glucose tolerance in the n0 STZ diabetic model. However, this would be too time-consuming and uneconomical. Thus only four sub-fractions (which together covered the whole range of compounds present in the hexane extract) were chosen for OGTT.

5.3.1 Materials and method

TLC of plant extracts

The four sub-fractions of hexane extract chosen for the study were M2C, M2D, M2E and M2I as these contained, between them, all the components of the hexane extract. In order to determine the dose of each sub-fraction used for the *in vivo* testing, an estimation of the concentration of each extract as compared to that of the whole crude hexane extract was carried out. Since 2.2302g of dried hexane extract was obtained from 7.8L karela juice, 100ml of karela juice should give 28.6mg dried hexane residue. The crude hexane extract residue was reconstituted in hexane solvent to give a concentration of 50µg/µl. Each of the four sub-fractions was reconstituted in hexane solvent to give a concentration of 10µg/µl. On a RP-18 plate (20cm ×

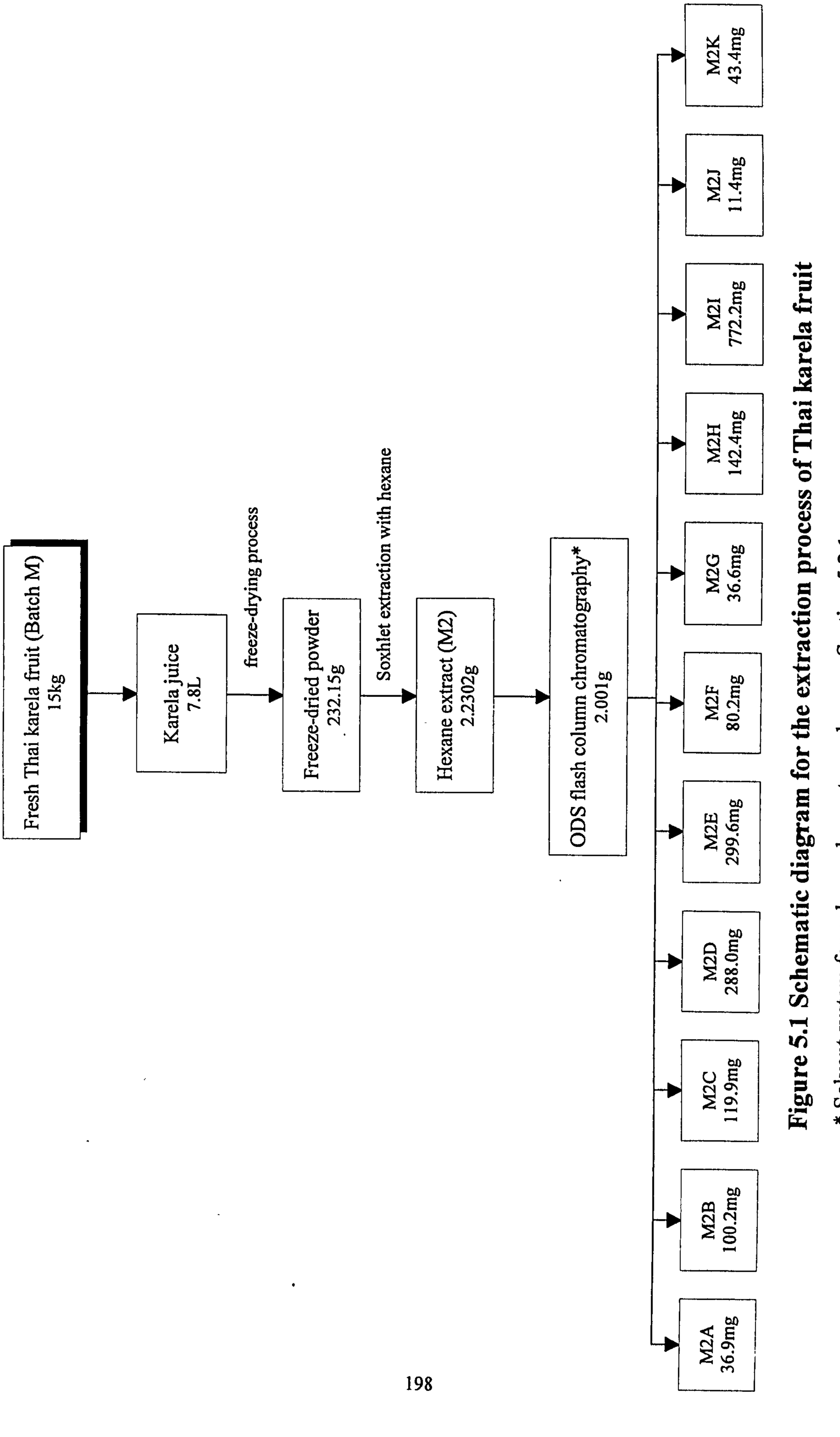
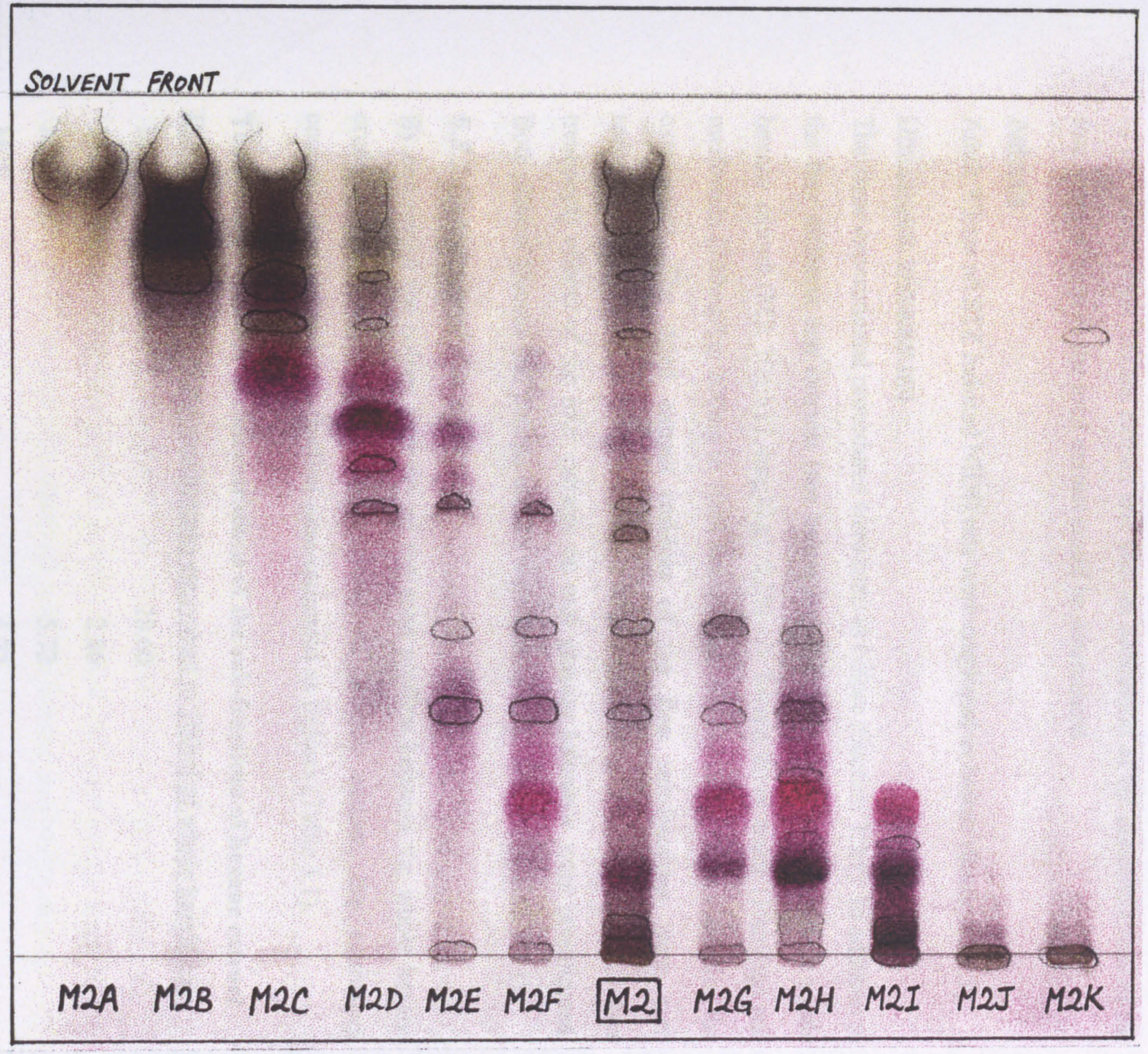


Figure 5.1 Schematic diagram for the extraction process of Thai karela fruit

* Solvent system for column chromatography - see Section 5.2.1



RP-18 F_{254S} plate

Application : 10µl

Solvent system -
MeOH : CH_3CN
2 : 1

Detection -
i) UV shortwave (254nm) (zones marked by pencil)
ii) Anisaldehyde spraying agent

Figure 5.2: TLC zone profiles of sub-fractions of Thai karela hexane extract (M2)

20cm), 10µl of crude hexane extract and 2, 5, 10 and 20µl of each of the four sub-fractions of hexane extract were applied. The plate was developed using a solvent system of MeOH: CH_3CN (2:1) and after development, it was sprayed with 0.5% anisaldehyde in methanol: glacial acetic acid: concentrated sulphuric acid (85:10:5) with subsequent heating at 100^0C for 10 minutes. The colour intensity of the zone profile of the four sub-fractions (at different concentrations) was compared with that of the whole crude hexane extract and thus an estimation of the concentrations of the four sub-fractions in the total extract could be determined.

Animals

Adult Wistar n0 STZ induced NIDD rats were employed as before (Section 3.2.1).

Oral glucose tolerance test

The same experimental procedures were used as before (Section 3.2.1). In addition to the four different test extracts (see below for dosages tested) and the whole crude hexane extract (M2; 10ml(11.4mg)/kg), 0.3% v/v Tween 80 in water (10ml/kg) and metformin (200mg/kg; 200mg in 10ml water) were used as controls for validating the experiment. The dried extract residues of the four sub-fractions were each reconstituted in 100ml of 0.3% v/v Tween 80 prior to testing. The cumulative (4 days treatment) effects of the four extracts on oral glucose tolerance were investigated. Both plasma glucose and plasma insulin were measured as before (Section 3.2.1).

5.3.2 Results and discussion

By comparing the colour intensity of the zone profiles between the whole hexane crude extract and the four sub-fractions of hexane extract, the equivalent concentrations of each sub-fraction were estimated as follows (Table 5.1).

Table 5.1: Estimation of concentrations of the sub-fractions of hexane extract

Extracts	Dry weight (mg) equivalent to 100ml of whole karela juice
M2	28.60
M2C	2.86
M2D	5.72
M2E	5.72
M2I	11.44

Instead of testing the above five extracts at concentrations equivalent to that of the whole karela juice, in the OGTT, they were tested at four times more concentrated dosages (Table 5.2). This was to make sure that any active constituents present would be concentrated enough to produce positive effect on oral glucose tolerance, taking into account that there might be more than one active constituent in the hexane extract, and the fractions would be weaker than the total extract.

Table 5.2: Dosages of sub-fractions of hexane extract tested in OGTT

Extracts	Dosages used in OGTT (in terms of 10ml/kg body weight)
M2	11.44mg
M2C	1.14mg
M2D	2.29mg
M2E	2.29mg
M2I	4.58mg

The negative result for 0.3% v/v Tween 80 and the positive effect of metformin on oral glucose tolerance validated the experimental model (results not shown). The activity of the whole crude hexane extract (M2) on oral glucose tolerance was also confirmed (Appendix 7). From the results of the OGTT (Fig. 5.3), both extracts M2C and M2I significantly improved oral glucose tolerance after four days of treatment, with mean ΔG[1] reduced by 22% ($p = 0.049$) and 17% ($p = 0.082$) respectively. However, extract M2D had no effect on oral glucose tolerance, whereas extract M2E increased mean plasma glucose at all time points after four days of treatment, though none of them reached statistical significance. Despite the positive effect of both extracts M2C and M2I on oral glucose tolerance, neither of them had any significant effect on plasma insulin (Table 5.3).

It is important to note that the effects of the two active fractions M2C and M2I (Fig. 5.3) were much weaker than that of the whole crude hexane extract (Appendix 7). This is possibly due to synergistic or additive effect of the active components. In fact, this weakening effect was also observed with the hexane and water extracts (Fig.

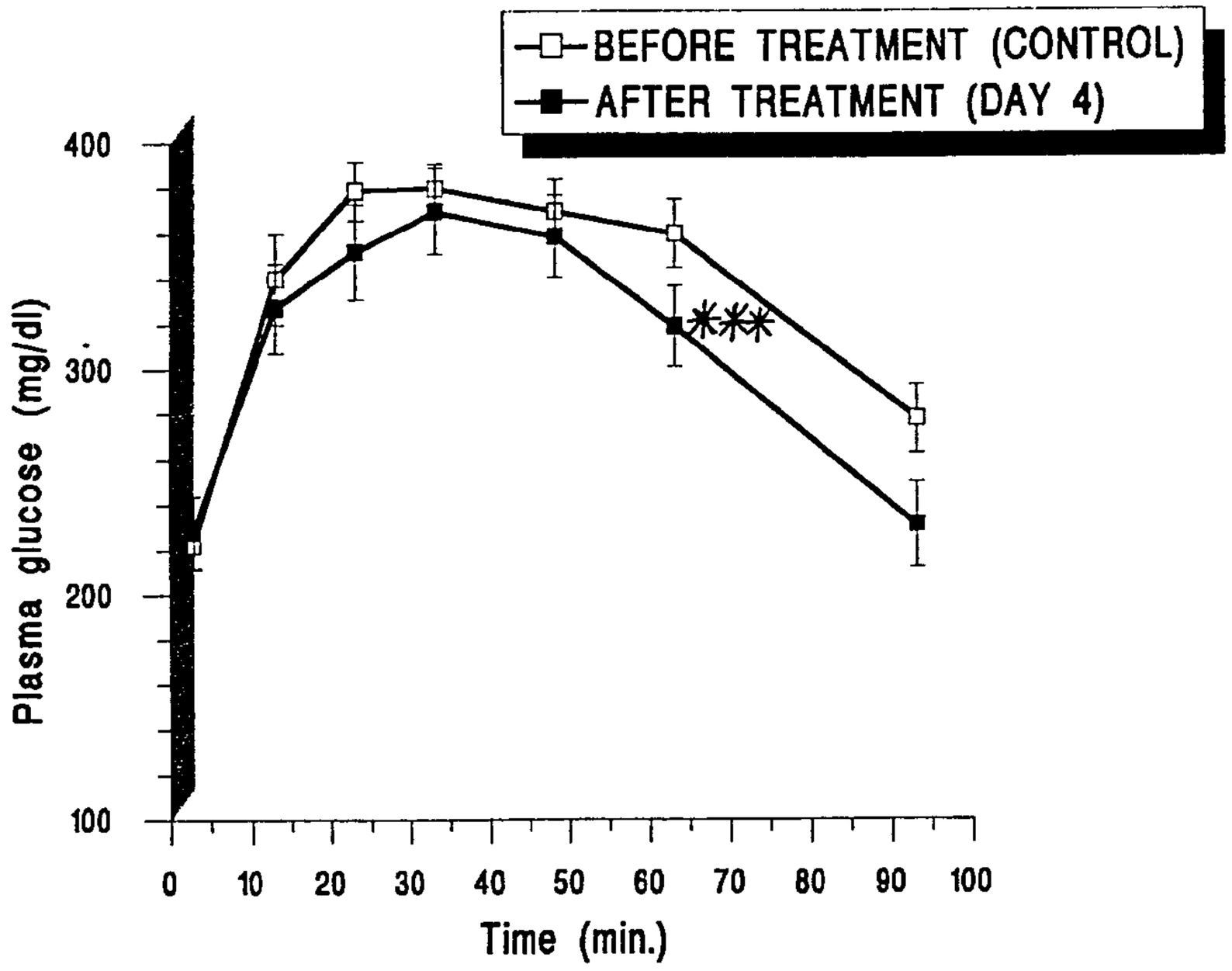
[1] ΔG = the sum of the increase in plasma glucose at all time points compared with time 0

Table 5.3: Effect of orally administered hexane extract sub-fractions M2C (10ml(1.14mg)/kg body weight) and M2I (10ml(4.58mg)/kg body weight) on plasma insulin in response to oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6) after four days of treatment. Glucose challenge was given at time 0; karela extracts were given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Extract M2C							
Control Day (n=6)	145.3 ± 20.0	243.2 ± 36.6	226.3 ± 39.2	251.0 ± 81.5	161.2 ± 37.4	188.8 ± 42.4	220.5 ± 46.5
Day 4 (n=6)	150.8 ± 29.9	260.0 ± 46.5	164.8 ± 26.4	179.3 ± 36.9	178.7 ± 6.4	142.5 ± 20.1	203.5 ± 23.7
Extract M2I							
Control Day (n=6)	159.3 ± 45.8	214.8 ± 38.2	195.0 ± 29.5	186.0 ± 29.0	150.3 ± 9.5	127.8 ± 12.8	151.8 ± 12.0
Day 4 (n=6)	93.0 ± 10.3	171.8 ± 24.2	210.7 ± 36.7	163.8 ± 22.9	158.3 ± 23.4	151.0 ± 15.9	158.3 ± 21.7

a)



b)

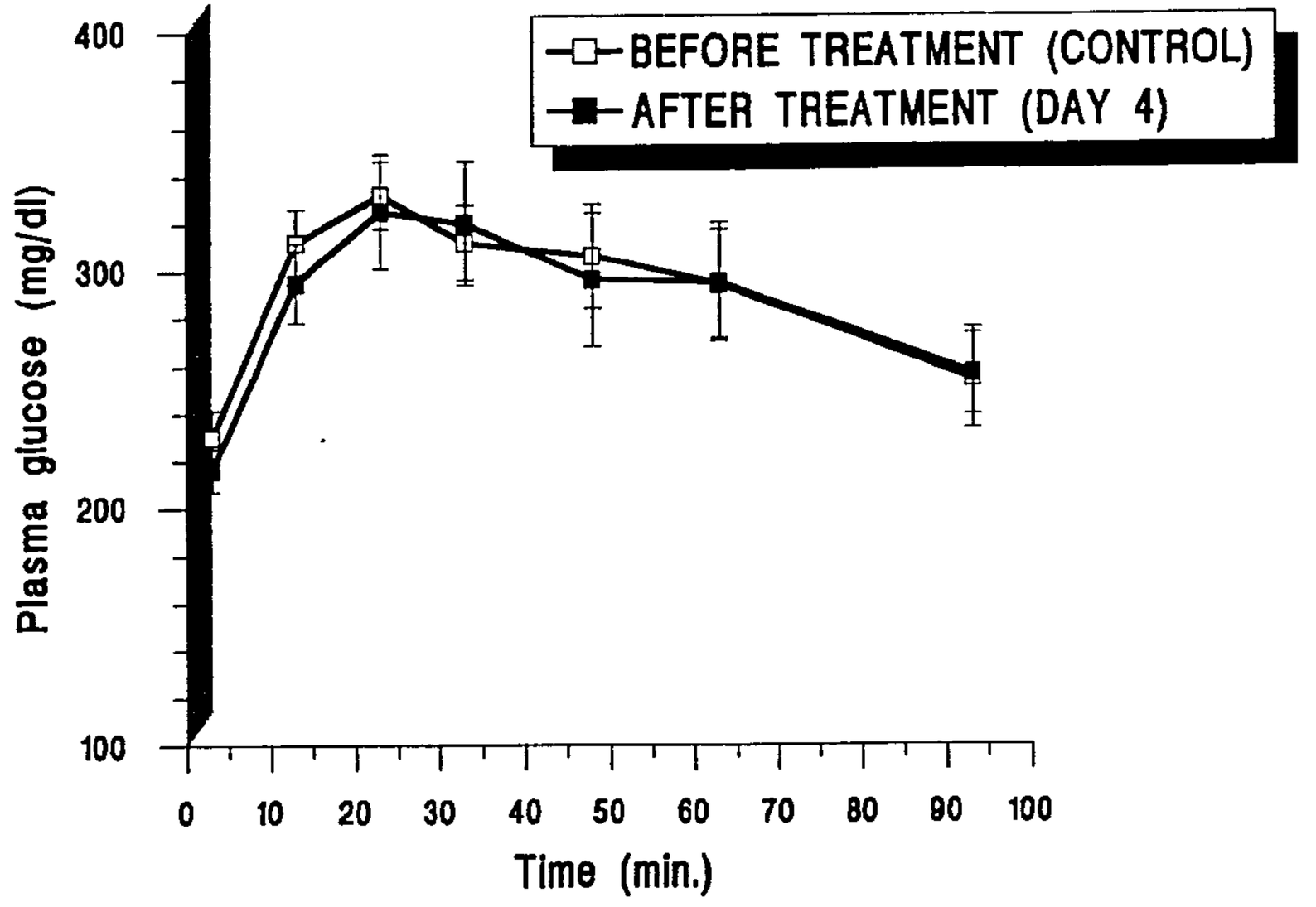
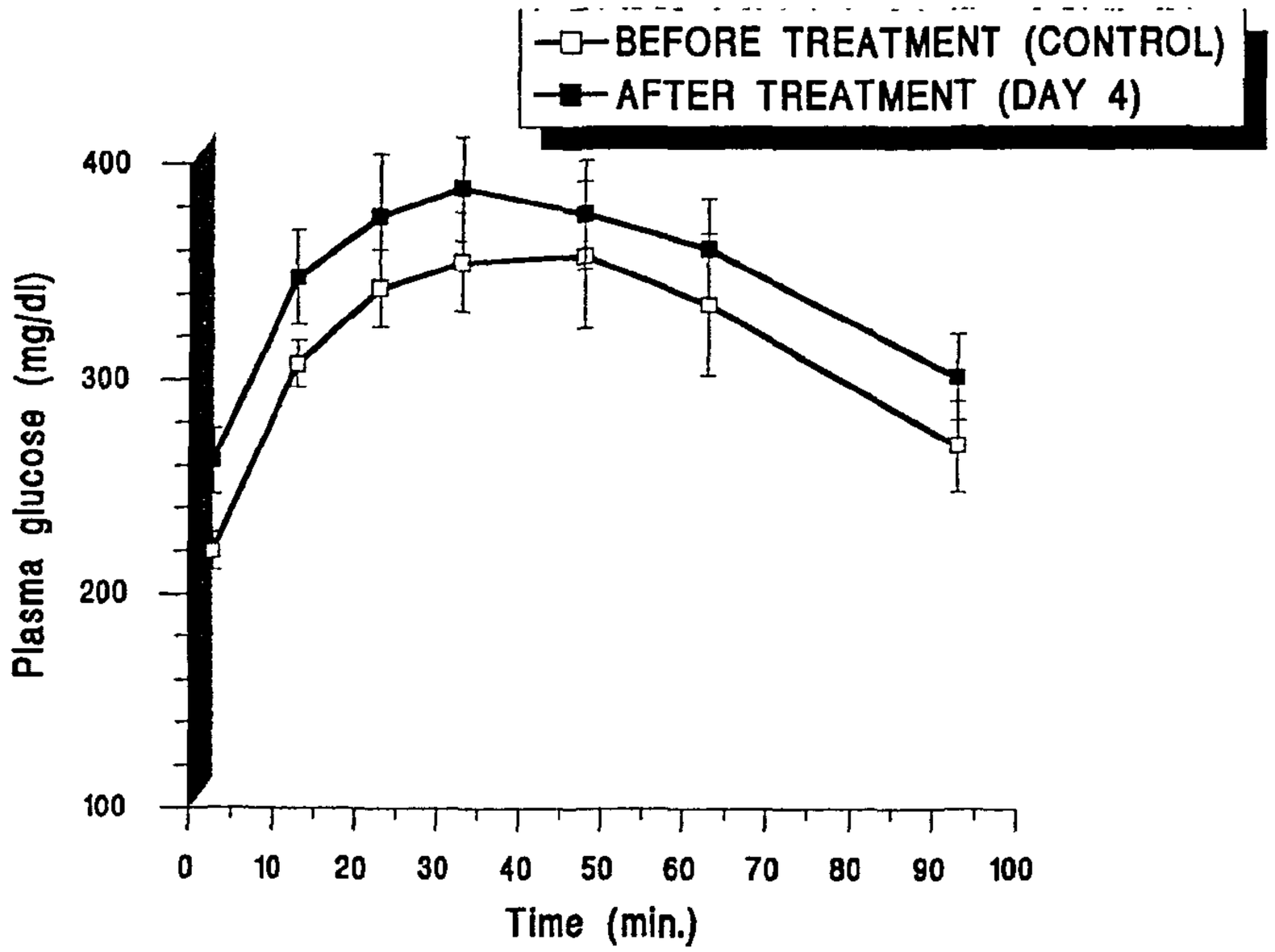


Figure 5.3: Effect of orally administered *a) M2C* (10ml(1.14mg)/kg body weight) and *b) M2D* (10ml(2.29mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min before glucose. Values of plasma glucose are mean ± SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control, by Student's paired t-tests. cont.)

c)



d)

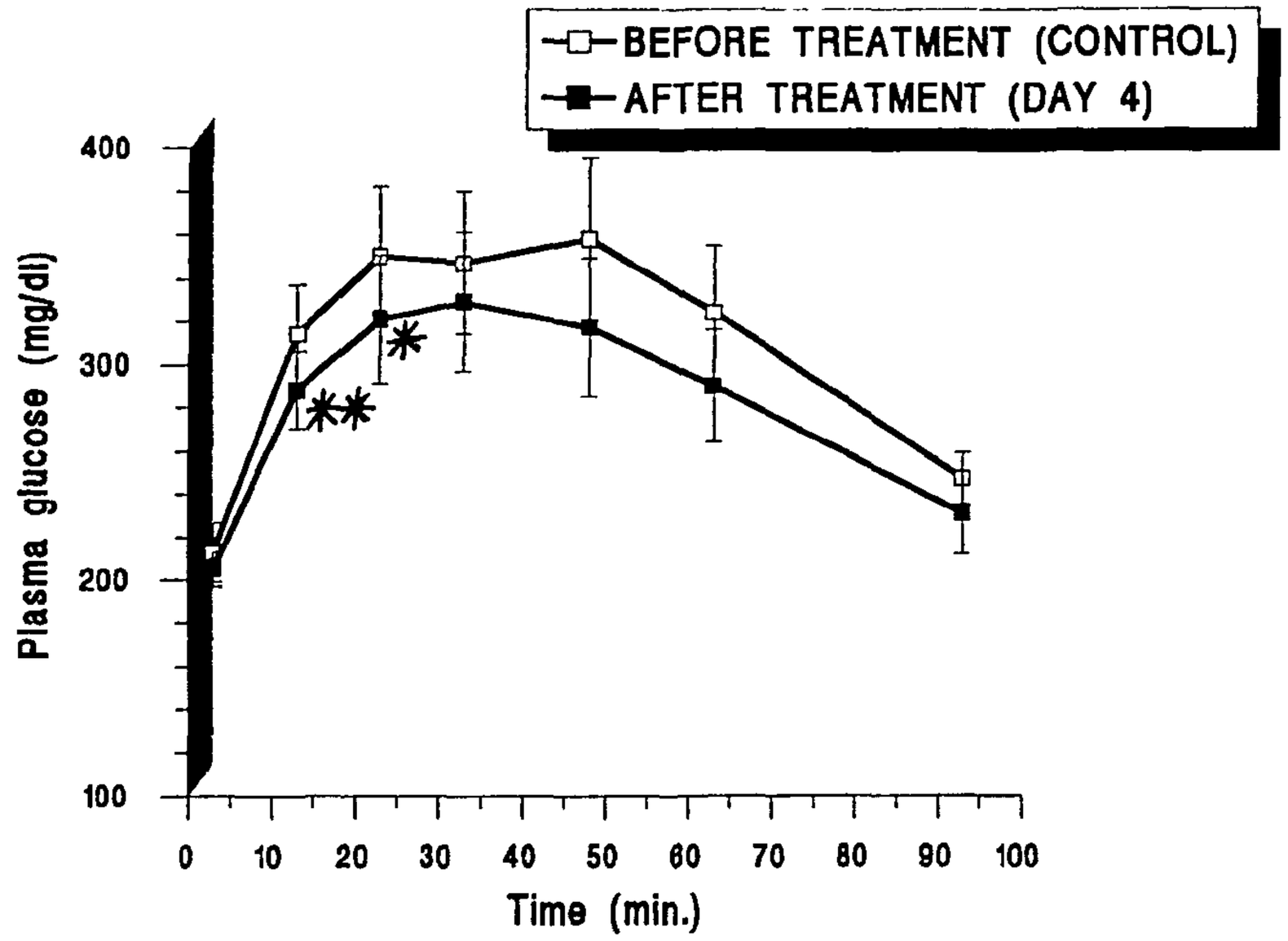


Figure 5.3(cont.): Effect of orally administered *c) M2E* (10ml(2.29mg)/kg body weight) and *d) M2I* (10ml(4.58mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; karela extracts were given 30 min before glucose. Values of plasma glucose are mean ± SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control, by Student's paired t-tests.

3.8) as compared to the whole karela juice (Fig. 3.3).

5.4 Fractionation of extract M2I

5.4.1 First stage of fractionation

5.4.1(a) Materials and methods

In order to further fractionate the extract M2I, a suitable chromatographic system had to be sought. Since hexane extract should contain relatively non-polar compounds, separation using a lipophilic Sephadex® LH-20 (particle size 25-100μ, Sigma) column was initially employed. A small sample of extract M2I (16.7mg) was applied to a LH-20 column (19cm in length × 17mm internal diameter) with the mobile system of MeOH : $CHCl_3$ (1:1). However, good separation of components was not achieved using this system.

Then further trials were run using silica as the adsorbent, with various non-polar solvents such as C_6H_{14}, $CHCl_3$ at different concentrations. It was found that better separations of components were achieved using silica than LH-20 as adsorbent. Thus a silica column was finally used to further fractionate hexane extract M2I.

Approximately 150g silica powder C60-H (particle size 40-60μm, Rhone-Poulenc) was used to pack a column (35cm in length × 26mm internal diameter, with space in column of 30ml). The column was conditioned with C_6H_{14} and flash chromatography was used where nitrogen gas was employed. The dried extract M2I (600mg), premixed with minimum amount of hexane solvent, was applied to the top of the silica column. The column was then eluted by gradient elution of increasing polarity:

i) C_6H_{14} (300ml) **f1-30*

ii) C_6H_{14} : $CHCl_3$ = 80:20 (300ml) *f31-57*

iii) C_6H_{14} : $CHCl_3$ = 60:40 (200ml) *f58-74*

iv) C_6H_{14} : $CHCl_3$ = 40:60 (200ml) *f75-91*

v) C_6H_{14} : $CHCl_3$ = 20:80 (300ml) *f92-112*

vi) $CHCl_3$ (1000ml) *f113-183*

vii) $CHCl_3$: MeOH = 1:1 (200ml) *f184-197*

viii) MeOH (100ml) *f198-200*

**f* = fractions collected

5.4.1(b) Results and discussion

From the silica flash column, 200 sub-fractions were collected. TLC zone profile of various sub-fractions are shown on Fig. 5.4 and the sub-fractions were pooled together as in Table 5.4. From the results, one of the main component (red-brown spot with R_f 0.13-0.14; Fig. 5.4) in extract M2I resided in fractions 169-193 (M2It; Table 5.4). Further fractionation of hexane extract M2It was carried out in order to isolate this compound. Since this compound did not quench UV light, it is unlikely to have an aromatic structure. An early prediction of this compound was that it was likely to be either steroidal or triterpenoid, as it reacted with anisaldehyde (a spraying agent for detection of sugars, steroids or triterpenes) to give a red/dark pink colour.

5.4.2 Second stage of fractionation (fractionation of extract M2It)

5.4.2(a) Materials and method

Hexane extract M2It (210mg) was further fractionated by putting through another silica column (particle size 18-32μm, ICN Biomedicals; 23cm in length × 17mm internal diameter) and the column was eluted with the following solvents of increasing polarity:

i) $CHCl_3$ *f1-14*

ii) $CHCl_3$: EtOAc (ethyl acetate) = 3:1 *f15-17*

iii) $CHCl_3$: EtOAc = 2:1 *f18*

iv) $CHCl_3$: EtOAc = 1:1 *f19*

v) EtOAc *f20-24*

vi) EtOAc : MeOH = 1:1 *f25-27*

vii) MeOH *f28-29*

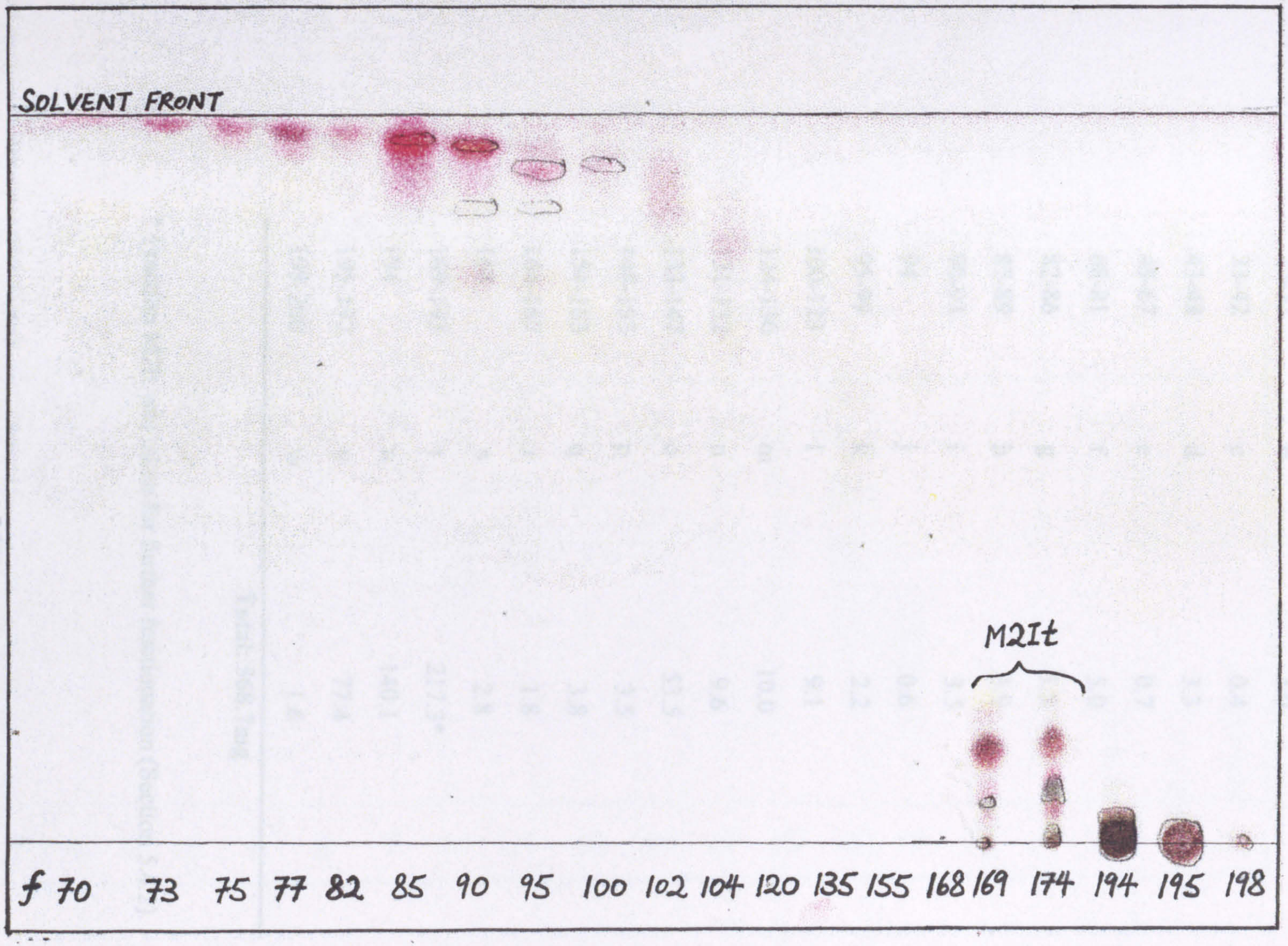
5.4.2(b) Results

Figure 5.5 shows the TLC zone profile of sub-fractions of extract M2It. The main component of interest (dark red spot; R_f 0.58) resided in fractions 9-11 (123.9mg).

5.4.3 Third stage of fractionation (further fractionation of extract M2It)

5.4.3(a) Materials and method

Fractions 9-11 (123.9mg) were further fractionated by putting through another silica column (particle size 18-32μm, ICN Biomedicals; 23cm in length × 17mm internal



Silica gel 60 F_{254} plate

Application : 10µl

Solvent system - $CHCl_3$

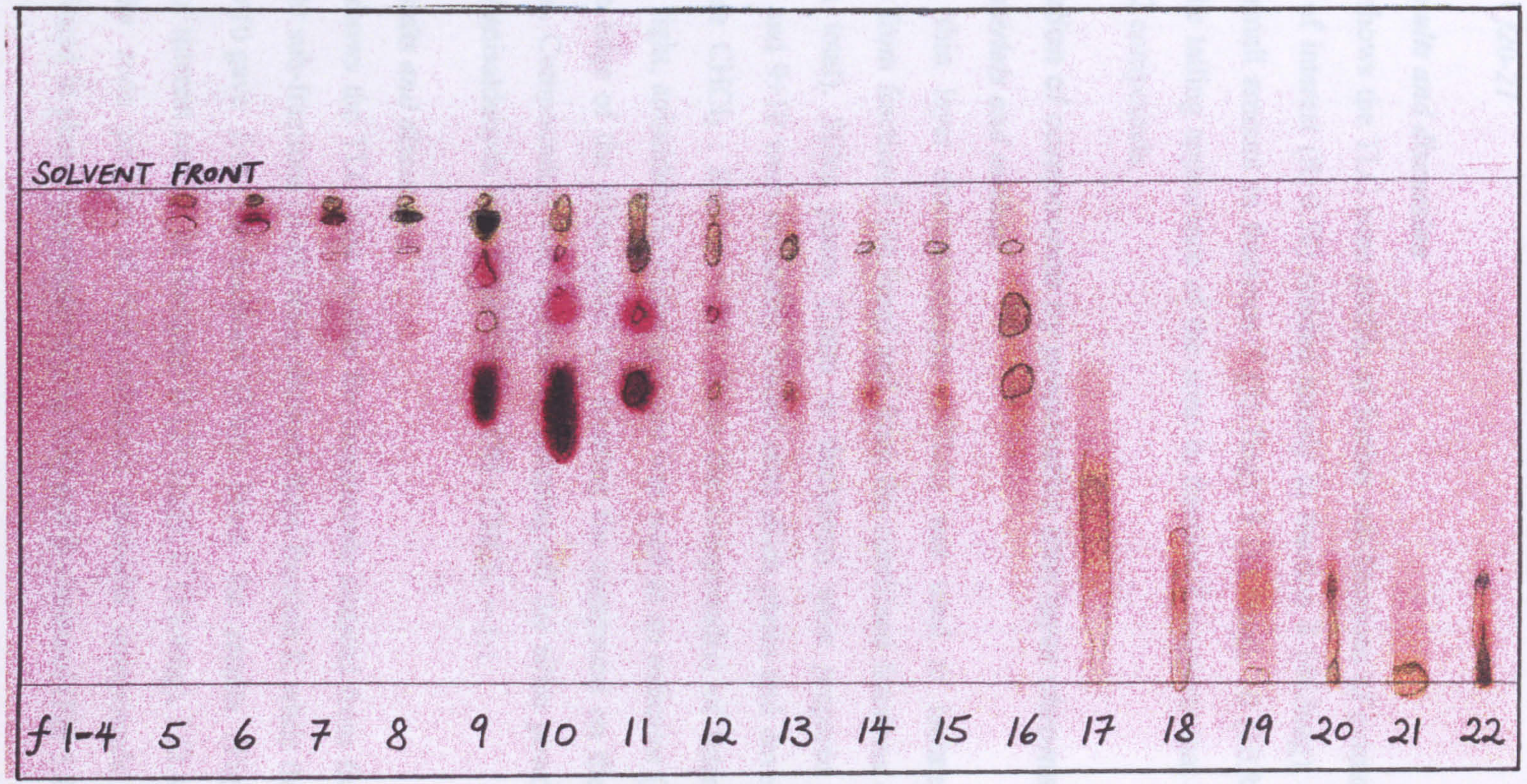
Detection -
i) UV shortwave (254nm) (zones marked by pencil)
ii) Anisaldehyde spraying agent

Figure 5.4: TLC zone profiles of sub-fractions of Thai karela hexane extract (M2I)

Table 5.4 Sub-fractions of hexane extract M2I

Fractions	Codes	Weight (mg)
1-24	a	10.1
25-32	b	3.0
33-42	c	0.4
43-48	d	3.3
49-67	e	0.7
68-81	f	5.0
82-86	g	5.5
87-89	h	3.9
90-93	i	3.5
94	j	0.6
95-99	k	2.2
100-123	l	9.1
124-130	m	10.0
131-132	n	9.6
133-147	o	53.5
148-153	p	3.5
154-163	q	3.8
164-167	r	1.8
168	s	2.8
169-193	t	217.3*
194	u	140.1
195-197	v	77.4
198-200	w	1.6
		Total: 568.7mg

* Fraction M2It was taken for further fractionation (Section 5.4.2)



Silica gel 60 F_{254} plate

Application : 10µl

Solvent system -
$CHCl_3$: EtOAc = 3:1

Detection -
i) UV shortwave (254nm)
(zones marked by pencil)
ii) Anisaldehyde spraying agent

Figure 5.5: TLC zone profiles of sub-fractions of Thai karela hexane extract (M2It)

diameter) and the column was eluted with the following solvents of increasing polarity:

i) $CHCl_3$ *f1-14*

ii) $CHCl_3$: EtOAc = 1:1 *f15-18*

iii) EtOAc *f19*

iv) MeOH *f20-21*

5.4.3(b) Results and discussion

Figure 5.6 shows the TLC zone profile of further sub-fractions of extract M2It. The compound of interest (R_f 0.58) resided mainly in fraction 8 (46.9mg), though also present in small amount in fractions 7 (33.7mg), 9 (11.0mg), 10 (10.8mg) and 11 (2.1mg). The tailing appearance of the zone in fraction 8 suggests that it may be a mixture of 2 compounds.

5.4.4 Isolation of compounds by preparative thin layer chromatography

5.4.4(a) Materials and method

Preparative thin layer chromatography (PTLC) was used to isolate individual compounds from fraction 8 (46.9mg) and from the combined fractions of 9 and 10 (21.8mg in total). Silica plates (1mm in thickness) were prepared for PTLC. Fractions 8 and 9+10 were separately loaded onto silica plates and developed using mobile phase $CHCl_3$: EtOAc = 3:1. Since the compound(s) of interest did not quench UV light, anisaldehyde spraying agent was used as an indicator (by spraying only on one edge of the silica plate) for locating the compound on the plate after development. Compound(s) was isolated by scraping off the silica powder at zones indicated by anisaldehyde and eluted with MeOH : $CHCl_3$ = 1:1.

5.4.4(b) Results and discussion

Figure 5.7 shows the TLC zone profile for compounds isolated from fractions 8, 9 and 10. Eight sub-fractions (A-H) were obtained from fraction 8, while the combined fractions 9+10 gave five sub-fractions (I-M). From the results (Fig. 5.7), the compound of interest resided in fractions B (27.1mg), C (15.4mg), I (2.4mg) and M (6.7mg). The zone profiles of two reference steroidal compounds which are commonly found in plants, stigmasterol (Fig 5.8) and β-sitosterol (Fig 5.8) were also

Silica gel 60 F_{254} plate

Application : 10µl

Solvent system -
$CHCl_3$: EtOAc = 3:1

Detection -
i) UV shortwave (254nm)
(zones marked by pencil)
ii) Anisaldehyde spraying
agent

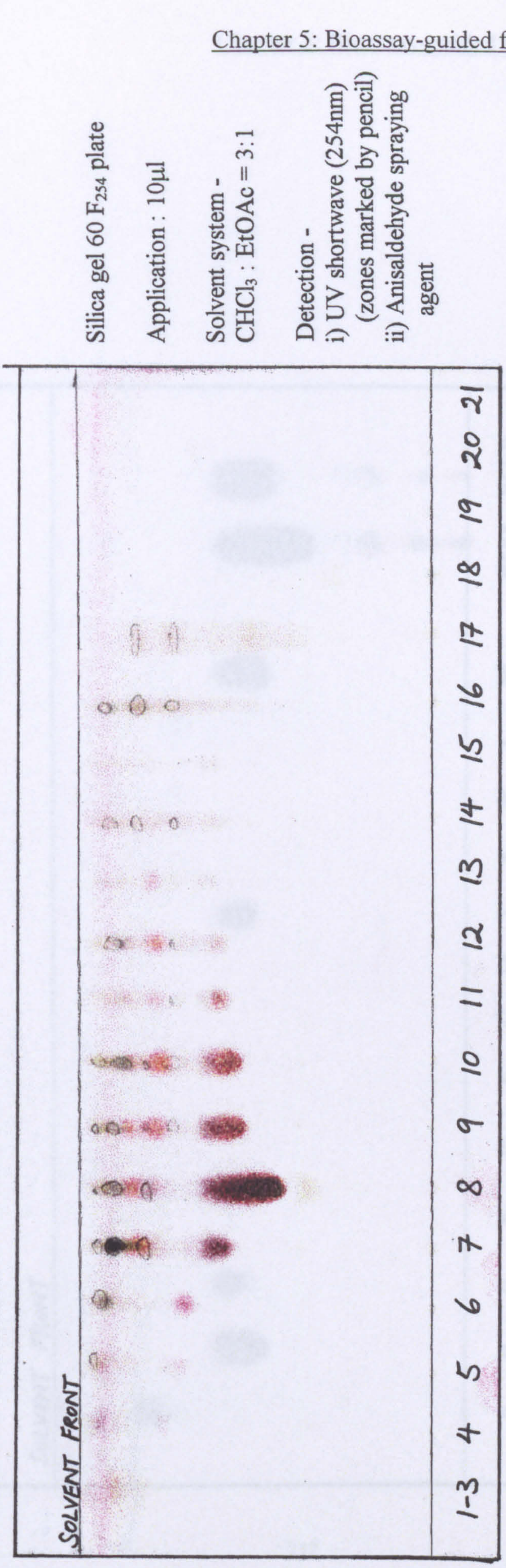
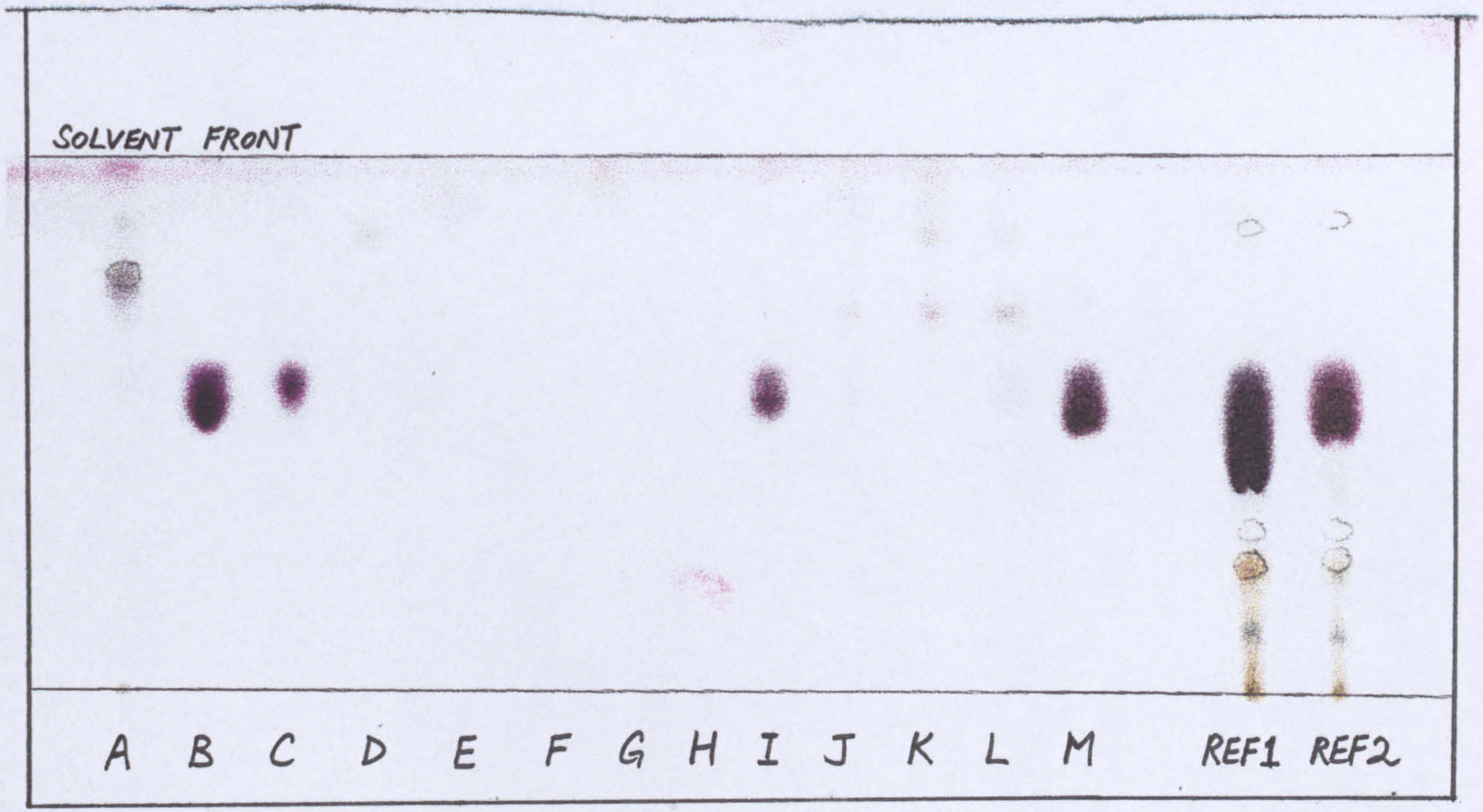


Figure 5.6: TLC zone profiles of further sub-fractions of Thai karela hexane extract (M2It)



Silica gel 60 F_{254} plate

Application : 10µl

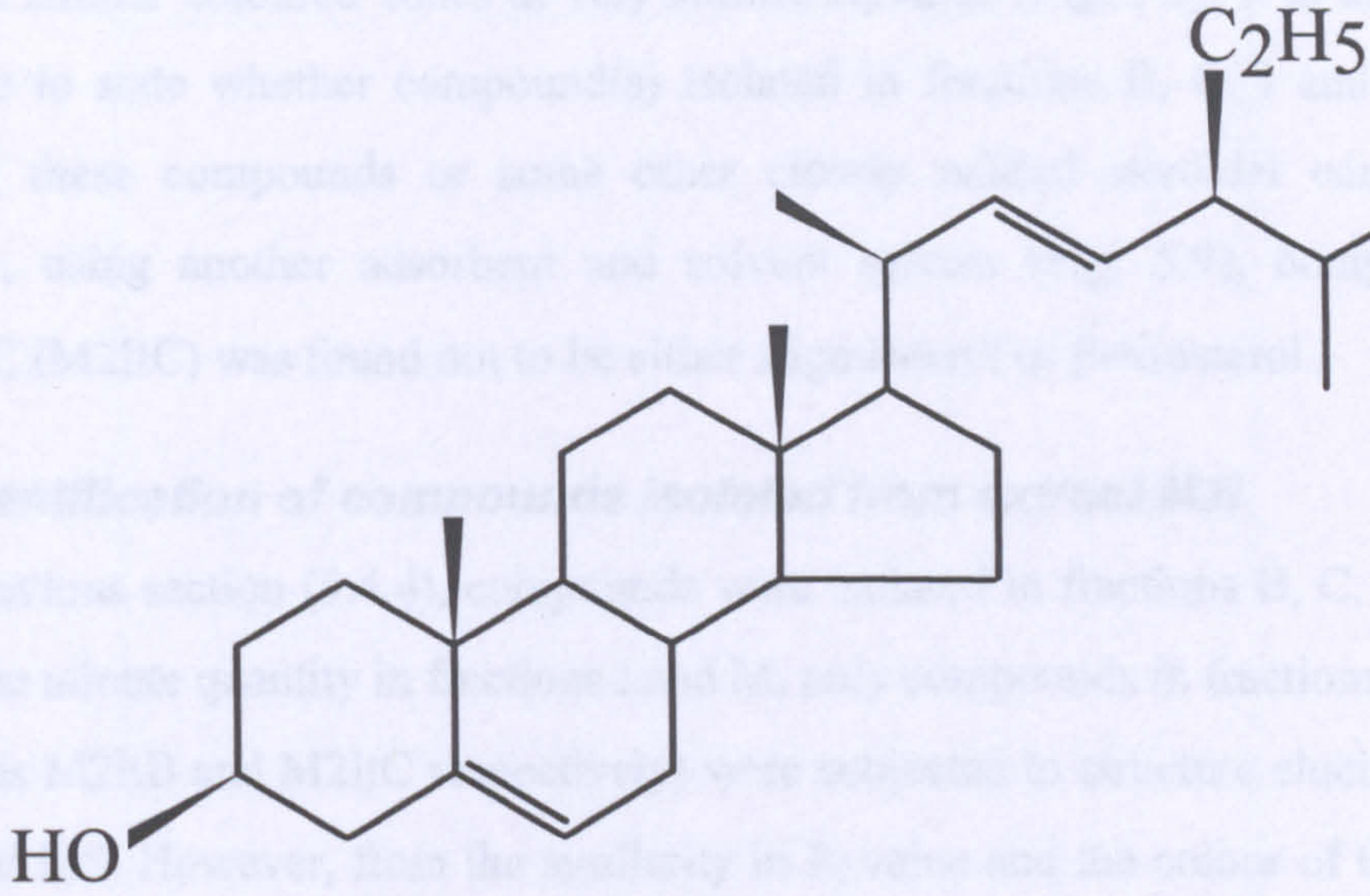
Solvent system -
$CHCl_3$: EtOAc = 3:1

Detection -
i) UV shortwave (254nm)
(zones marked by pencil)
ii) Anisaldehyde spraying agent

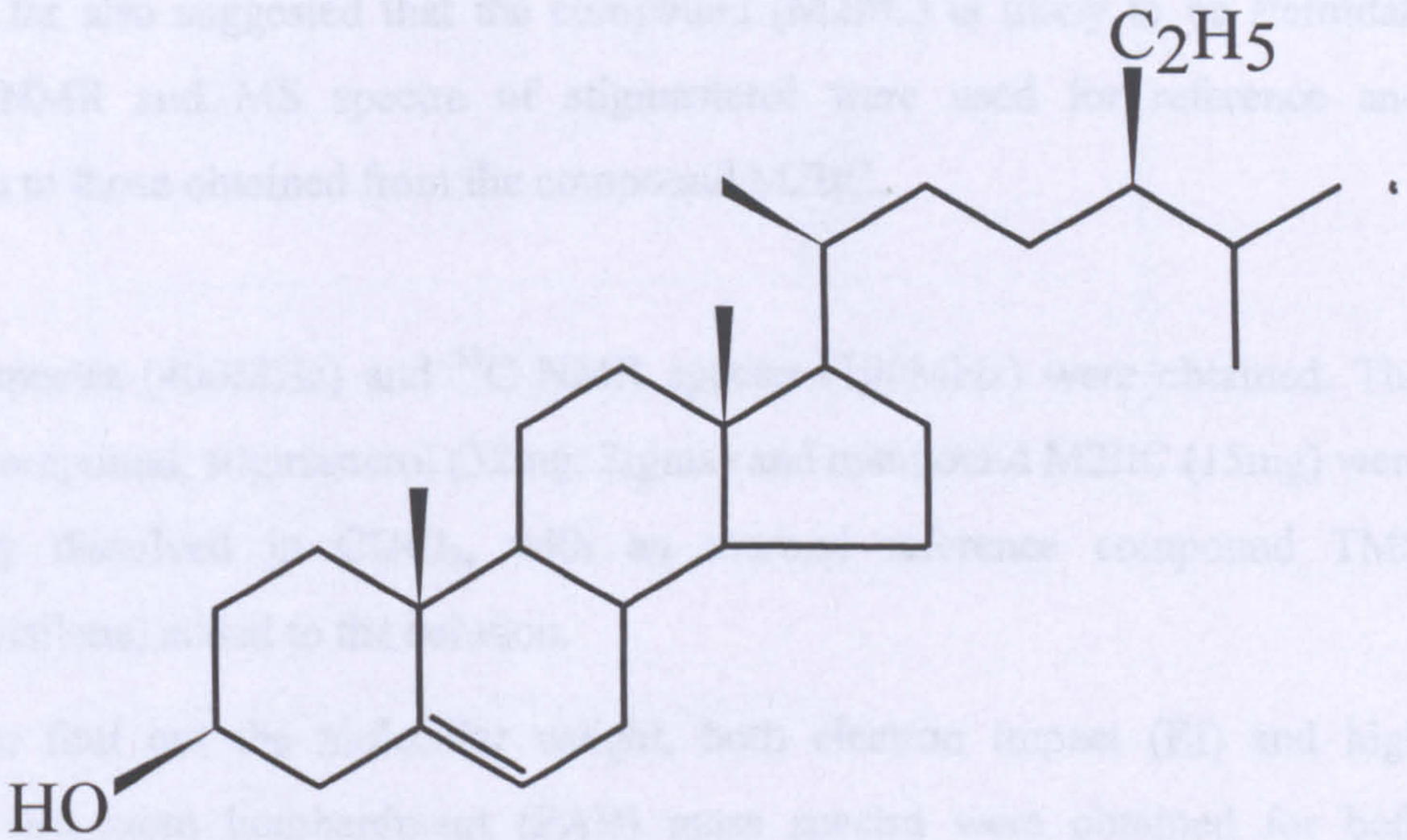
Figure 5.7: TLC zone profiles of further sub-fractions of Thai karela hexane extract (M2It)

Keys: REF1 = stigmasterol (Sigma; about 80% pure)

REF2 = β-sitosterol (Sigma; about 80% pure)



Stigmasterol ($C_{29}H_{48}O$; m.wt. 412)



β-Sitosterol ($C_{29}H_{50}O$; m.wt. 414)

Figure 5.8: Two steroidal compounds commonly found in plants

used for comparison. However, due to the fact that both stigmasterol and β-sitosterol presented similar coloured zones at very similar R_f value (Fig. 5.7), it is difficult at this stage to state whether compound(s) isolated in fractions B, C, I and M were either of these compounds or some other closely related steroidal compounds. However, using another adsorbent and solvent system (Fig. 5.9), compound in fraction C (M2ItC) was found not to be either stigmasterol or β-sitosterol.

5.4.5 Identification of compounds isolated from extract M2I

In the previous section (5.4.4), compounds were isolated in fractions B, C, I and M. Due to the minute quantity in fractions I and M, only compounds in fractions B and C (known as M2ItB and M2ItC respectively) were subjected to structure elucidation by NMR and MS. However, from the similarity in R_f value and the colour of the zones, the two compounds in fractions I and M were likely to be the same ones as in fractions B and C.

5.4.5(a) Compound M2ItC

TLC zone profile (Fig. 5.7) shows that a single compound was present in fraction C. Results so far also suggested that the compound (M2ItC) is likely to be steroidal. Thus the NMR and MS spectra of stigmasterol were used for reference and comparison to those obtained from the compound M2ItC.

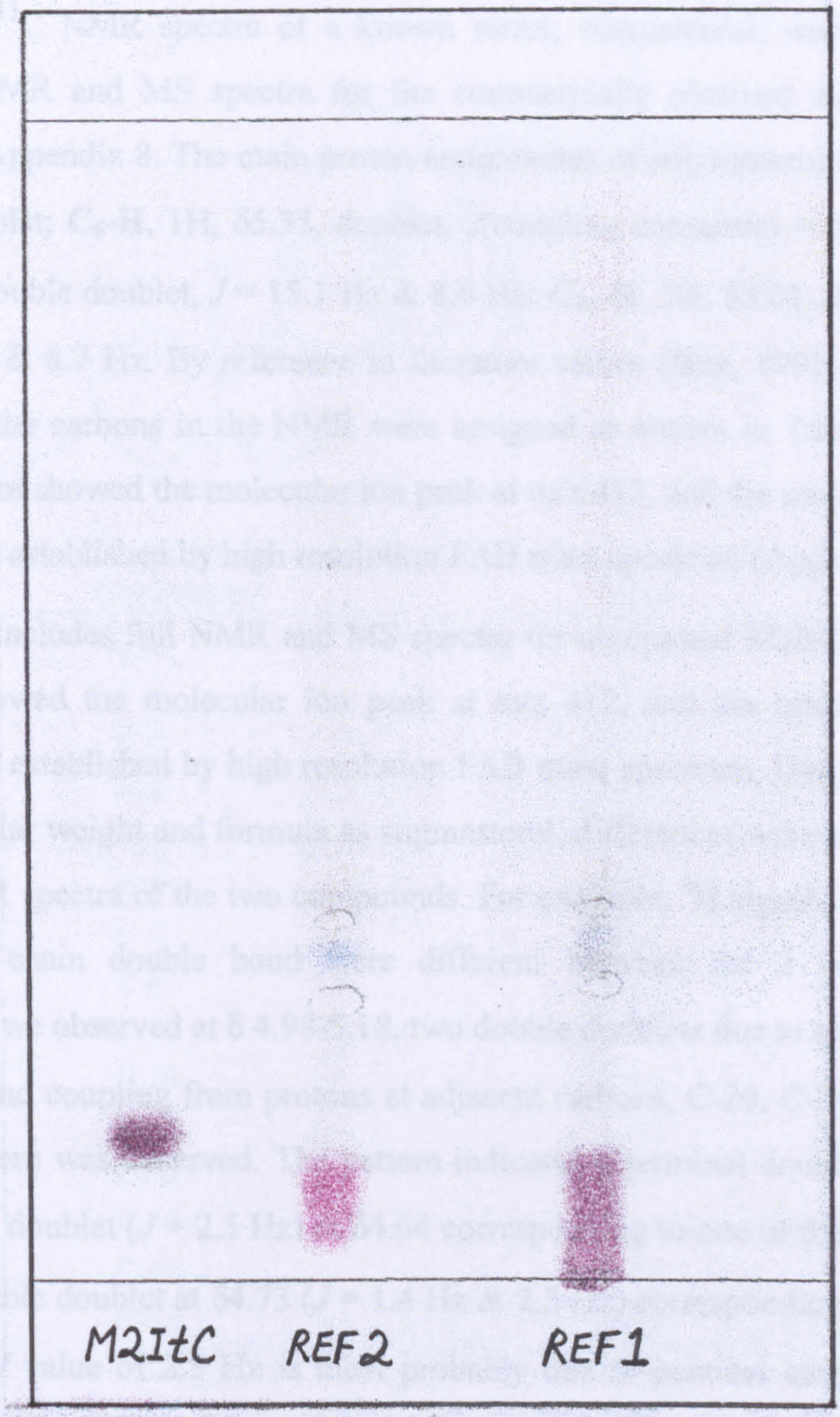
Method

^{1}H NMR spectra (400MHz) and ^{13}C NMR spectra (100MHz) were obtained. The reference compound, stigmasterol (32mg, Sigma) and compound M2ItC (15mg) were individually dissolved in $CDCl_3$, with an internal reference compound TMS (tetramethylsilane) added to the solution.

In order to find out the molecular weight, both electron impact (EI) and high resolution fast atom bombardment (FAB) mass spectra were obtained for both samples.

Results

The ^{1}H NMR spectrum for compound M2ItC showed a pattern characteristic of steroids whilst a proton signal at ~3.5ppm signified the possibility of a sterol. Thus



RP-18 F_{254S} plate

Application : 10µl

Solvent system - MeOH : CH_3CN (2:1)

Detection -

i) UV shortwave 254nm (zones marked by pencil)

ii) Anisaldehyde spraying agent

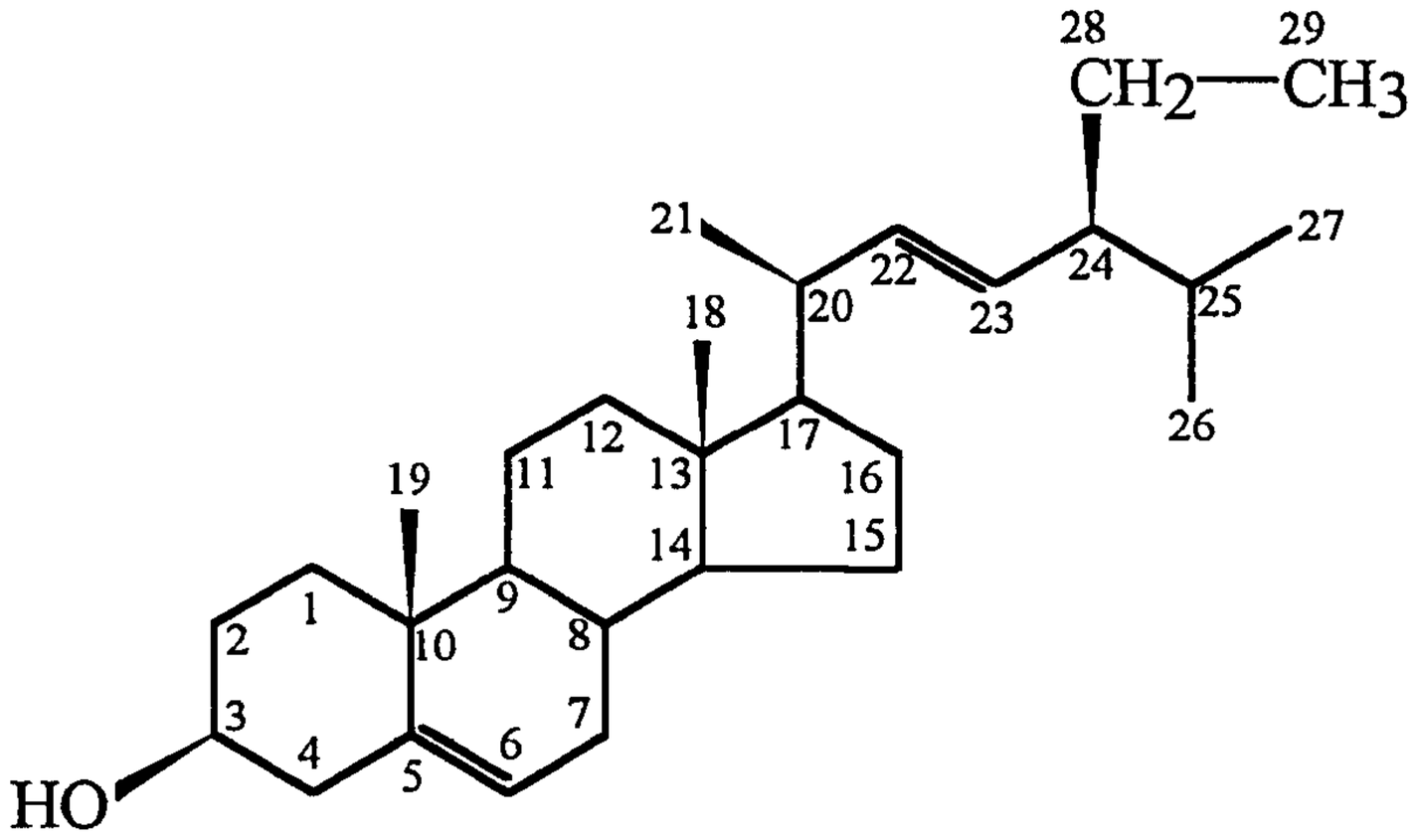
REF1 - stigmasterol; REF2 - β-sitosterol

Figure 5.9: TLC zone profiles of compound M2ItC and two reference steroidal compounds

the ^{1}H and ^{13}C NMR spectra of a known sterol, stigmasterol, were obtained for reference. NMR and MS spectra for the commercially obtained stigmasterol are included in Appendix 8. The main proton assignments of stigmasterol are: **C_3-H**, 1H, δ3.52, multiplet; **C_6-H**, 1H, δ5.35, doublet, *J*(coupling constants) = 5.3 Hz; **C_{22}-H**, 1H, δ5.14, double doublet, *J* = 15.1 Hz & 8.6 Hz; **C_{23}-H**, 1H, δ5.01, double doublet, *J* = 15.2 Hz & 8.7 Hz. By reference to literature values (Ikan, 1991), the chemical shifts of all the carbons in the NMR were assigned as shown in Table 5.5. The EI mass spectrum showed the molecular ion peak at m/z 412, and the molecular formula $C_{29}H_{48}O$ was established by high resolution FAB mass spectrum (Appendix 8).

Appendix 9 includes full NMR and MS spectra for compound M2ItC. The EI mass spectrum showed the molecular ion peak at m/z 412, and the molecular formula $C_{29}H_{48}O$ was established by high resolution FAB mass spectrum. Despite having the same molecular weight and formula as stigmasterol, differences were exhibited in ^{1}H and ^{13}C NMR spectra of the two compounds. For example, ^{1}H signals corresponding to the side chain double bond were different between the 2 compounds. In stigmasterol, we observed at δ 4.98-5.18, two double doublets due to transcoupling (*J* = 15.1 Hz) and coupling from protons at adjacent carbons, C-20, C-24. In M2ItC, a different pattern was observed. The pattern indicated a terminal double bond on the side chain. A doublet (*J* = 2.5 Hz) at δ4.64 corresponding to one of the protons at C-26, and a double doublet at δ4.73 (*J* = 1.4 Hz & 2.5 Hz) corresponding to the second proton. The *J* value of 2.5 Hz is most probably due to geminal coupling of the 2 protons on C-26, while the *J* value of 1.4 Hz may represent long-range coupling of one of these protons to that on C-24. In ^{13}C NMR spectrum, the chemical shifts are virtually identical between the 2 compounds, apart from those in the double bond region. The signals correspond to **C-5** (δ141.2) and **C-6** (δ122.1) in stigmasterol are also found in M2ItC (δ140.8 and δ121.8), indicating that a double bond exists between C-5 & C-6, same as stigmasterol. However, the other 2 signals in stigmasterol (δ 138.8 and 129.7) were very different from those in M2ItC (δ147.6 and δ111.4). This suggests that compound M2ItC was not stigmasterol, but very likely to be a closely related steroidal compound, possibly with the side chain double

Table 5.5: ^{13}C NMR ($CDCl_3$) spectral data for stigmasterol



Carbon no.	ppm
C-1	37.7
C-2	32.1
C-3	72.2
C-4	42.7
C-5	141.2
C-6	122.1
C-7	32.3
C-8	32.3
C-9	50.6
C-10	36.9
C-11	21.5
C-12	40.1
C-13	42.6
C-14	57.3
C-15	24.8
C-16	29.4
C-17	56.4
C-18	12.5
C-19	19.8
C-20	40.9
C-21	21.5
C-22	138.8
C-23	129.7
C-24	51.7
C-25	32.3
C-26	21.6
C-27	19.4
C-28	25.8
C-29	12.7

bond at a different position in agreement with the ^{1}H spectrum. In fact, the ^{13}C NMR spectrum for M2ItC showed the presence of 29 carbon signals in the molecule. The DEPT spectrum exhibited 5 methyls, 12 methylene and 8 methine signals, while the remaining four signals were due to the quaternary carbon atoms. On the basis of all the spectral evidences, the structure of M2ItC was formulated as 5,25-stigmastadienol, as confirmed with previous reported data (Ahmad *et al.*, 1993; Table 5.6). The main proton assignments are: **C_3-H**, 1H, δ3.53, multiplet; **C_6-H**, 1H, δ5.35, double doublet (J = 3.2 Hz & 2.1 Hz); **C_{26}-H**, 2H, δ4.64, doublet (J = 2.5 Hz) and δ4.73, double doublet (J = 2.5 Hz & 1.4 Hz); **C_{27}-H**, 3H, δ1.56, singlet.

5.4.5(b) Compound M2ItB

From the TLC zone profile (Fig. 5.7), there is only one spot in fraction B (27.1mg). However, the slightly tailing effect of the spot suggests that it may contain more than one compound, which would be confirmed by NMR.

5.4.5(b1) NMR

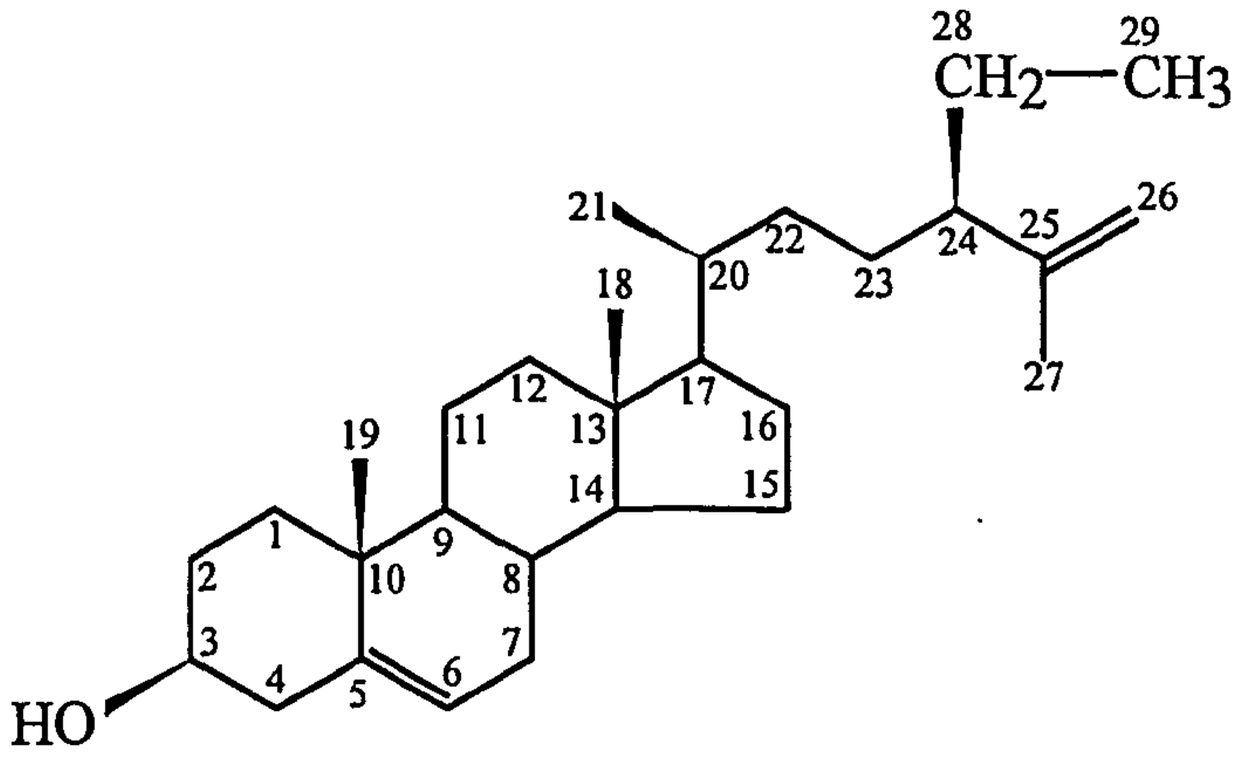
Method

^{1}H NMR spectra (400MHz) and ^{13}C NMR spectra (100MHz) were obtained. Compound M2ItB (27mg) was dissolved in $CDCl_3$, with an internal reference compound TMS added to the solution.

Results and discussion

^{1}H and ^{13}C NMR spectra of compound M2ItB are presented in Appendix 10. Results suggest that compound M2ItB is likely to be steroidal since both ^{1}H and ^{13}C spectral pattern matches those of sterols, like stigmasterol and 5,25-stigmastadienol. M2ItB is likely to be a mixture of two sterols because the ^{13}C spectrum gave a large number of carbons (>40). In fact, the ^{1}H spectrum for M2ItB appeared like the spectrum of 5,25-stigmastadienol overlaid with a second spectrum, e.g. extra signal at δ5.15 and a broader C_3-H signal (δ3.48-3.62). By examining the relative intensity of the double bond signals at δ5.15 and δ5.34, it appears that the mixture is about 1:1. Therefore, the spectral data suggests that M2ItB is likely to be a 1:1 mixture of two steroidal components. Hence attempts were made to separate the two components by TLC.

Table 5.6: ^{13}C NMR ($CDCl_3$) spectral data for compound M2ItC



5,25-stigmastadienol

Carbon no.	ppm (own results)*	ppm (literature)**
C-1	37.3	37.4
C-2	31.7	31.5
C-3	71.8	71.7
C-4	42.3	42.2
C-5	140.8	141.0
C-6	121.8	121.8
C-7	31.9	33.6
C-8	31.9	31.8
C-9	50.1	50.3
C-10	36.5	36.4
C-11	21.1	21.3
C-12	39.8	39.7
C-13	42.3	42.3
C-14	56.8	56.9
C-15	24.3	24.3
C-16	28.2	28.2
C-17	56.1	56.2
C-18	11.9	11.8
C-19	19.4	19.4
C-20	35.6	35.4
C-21	18.7	18.6
C-22	33.7	33.7
C-23	29.4	29.6
C-24	49.6	49.5
C-25	147.6	147.6
C-26	111.4	111.4
C-27	17.8	17.8
C-28	26.6	26.4
C-29	12.1	12.1

* Corrected ppm (the actual value on spectrum - 2.7ppm) as the $CDCl_3$ should have ppm at around 77. ** Ahmad *et al.*, 1993

5.4.5(b2) TLC

The following TLC systems were tried in order to separate the 2 steroidal compounds present in M2ItB, though none of them succeeded in separation of the mixture:

A) Using silica gel 60 F_{254} plate; with solvent systems:

   i) $CHCl_3$

   ii) $CHCl_3$: EtOAc = 4:1

   iii) $CHCl_3$: EtOAc = 3:1

   iv) multiple development with firstly $CHCl_3$, then $CHCl_3$: EtOAc (4:1), followed by $CHCl_3$: EtOAc (3:1)

B) Using RP-18 F_{254S} plate; with solvent system – MeOH: CH_3CN (2:1), a multiple development was tried (3 times and 4 times development).

Due to the failure in separating the mixture by TLC, attempt was made using GC-MS.

5.4.5(b3) GC-MS analysis

Materials and method

The instrument is JEOL AX505W.

Column : BP1 (supplied by SGE)

Phase	Bonded phase methyl siloxane
Phase thickness	0.25µm
Length	25m
Internal diameter	0.22mm

Carrier gas: Helium

Pressure	12psi
Flow	$1cm^3min^{-1}$

Temperature programme

Initial temperature	120°C
Initial time	2 min
Ramp rate	6°C min^{-1}
Final temperature	320°C
Final time	5 min

Injection: 0.2 µl on column

Three reference compounds were used: β-sitosterol, 5,25-stigmastadienol (M2ItC) and stigmasterol. Each of the samples (the reference compounds and M2ItB) was dissolved in 200µl $CHCl_3$ before analysis.

Results and discussion

The electron impact mass spectra for the three reference compounds are included in Appendix 11. Using this GC system, separation of sterols was achieved and the elution order of the three reference sterols (identified by their MS) was stigmasterol (34.4min after injection), followed by 5,25-stigmastadienol (34.8min), and finally β-sitosterol (35.1min), as shown in Appendix 11.

From the analysis, M2ItB was found to contain β-sitosterol and 5,25-stigmastadienol, i.e. a mixture of two sterols as predicted.

5.5 Effect of 5,25-stigmastadienol and β-sitosterol on oral glucose tolerance in n0 STZ NIDDM model

5.5.1 Introduction

In the previous section (5.4), extract M2I was shown to contain both 5,25-stigmastadienol and β-sitosterol as the main constituents. Thus the effects of both compounds on oral glucose tolerance were investigated in order to see if they are responsible for the anti-hyperglycaemic activity of the whole extract M2I.

5.5.2 Materials and method

Compounds tested

5,25-stigmastadienol (M2ItC, at doses of 10ml(1.3mg)/kg and 10ml(4.6mg)/kg) and β-sitosterol (10ml(3.1mg)/kg) were tested. Each compound was reconstituted in 100ml 0.3%v/v Tween 80 prior to testing.

Animals

Adult Wistar n0 STZ induced NIDD rats were employed as before (Section 3.2.1).

Oral glucose tolerance test

The same experimental procedures were used as before (Section 3.2.1). 0.3% v/v Tween 80 (10ml/kg) and metformin (200mg/kg; 200mg in 10ml water) were used as

controls for validating the experiment.

5.5.3 Results and discussion

From the results (Table 5.7), compound 5,25-stigmastadienol at dose of 1.3mg/kg improved oral glucose tolerance but none of the time points reached statistical significance. The dose tested was equivalent to the dose tested for the whole fraction M2I (Table 5.2) determined by quantitative TLC as described in Section 5.3. The lack of significant improvement in oral glucose tolerance may be due to the fact that the dose tested was too low. Thus both compounds, 5,25-stigmastadienol and β-sitosterol, were tested at about 3-4 times more concentrated dosages. From the results (Fig. 5.10), both 5,25-stigmastadienol and β-sitosterol significantly improved oral glucose tolerance (a reduction in ΔG by 51% ($p = 0.0015$) and 50% ($p = 0.0030$) respectively). It is interesting to note that in both cases, there was a significant increase in plasma glucose at time 0. No significant effect on plasma insulin was observed with either of the compounds (Table 5.8).

Here is another example of the synergistic or additive effect of the active components in karela (as previously discussed in Section 5.3.2). When 5,25-stigmastadienol was tested at an equivalent dose to that of whole hexane extract, it had no significant effect on oral glucose tolerance. It was necessary to increase the dose by about 3-4 times in order to see the positive effect.

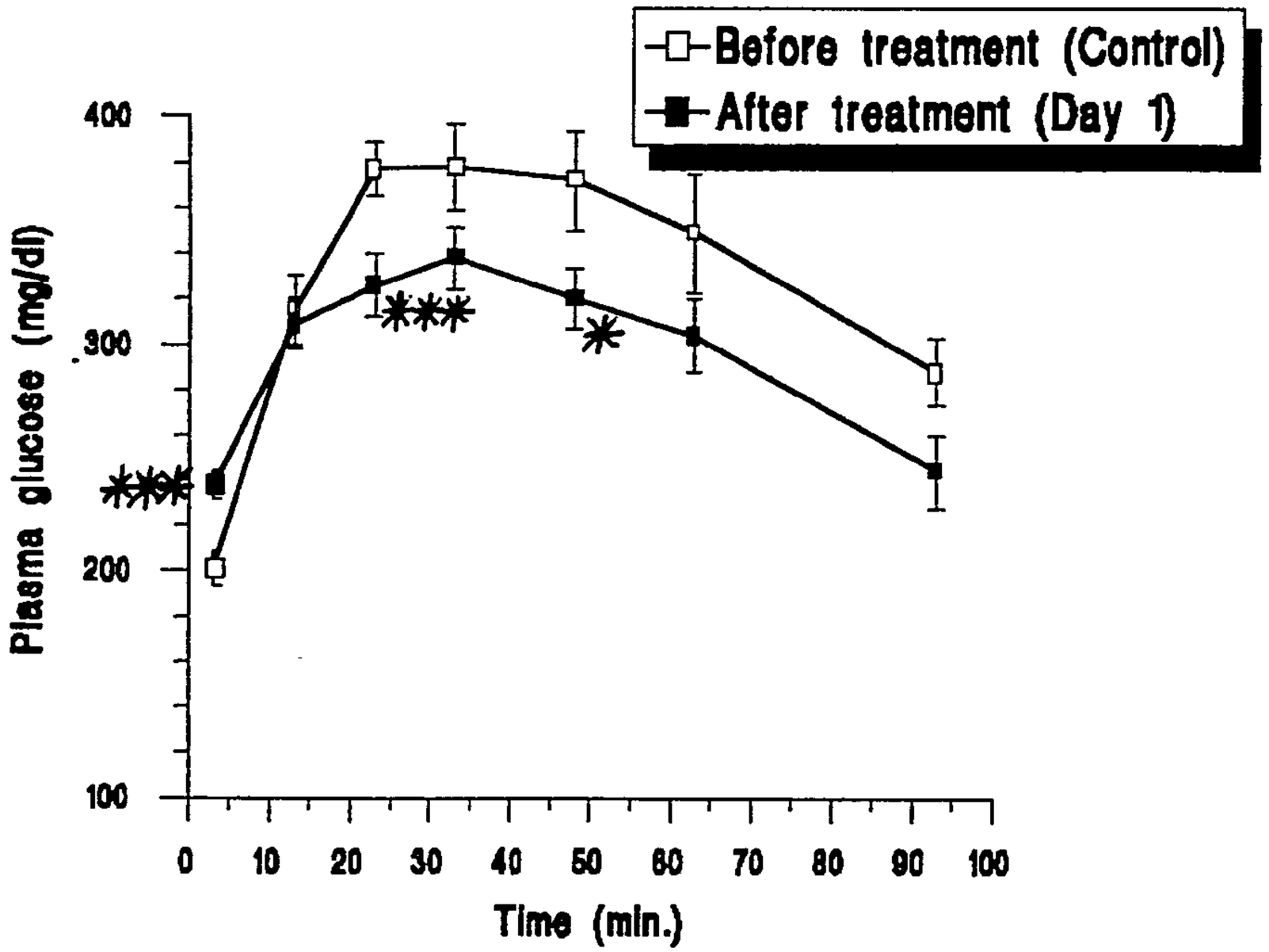
It is interesting to note that both 5,25-stigmastadienol and β-sitosterol are aglycones of charantin (Fig. 1.19; a 1:1 mixture of 5,25-stigmastadienol and β-sitosterol glucosides), a compound which was believed to be the active constituent in *Momordica charantia* (Section 1.4.3).

Table 5.7: Effect of orally administered 5,25-stigmastadienol (10ml(1.3mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; compound tested was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma glucose mg/dl (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Control Day (n=6)	230.0 ± 11.4	297.7 ± 18.1	332.2 ± 22.3	329.5 ± 17.4	301.8 ± 24.5	278.5 ± 26.4	243.7 ± 17.9
Day 1 (n=6)	202.5 ± 12.0	272.5 ± 12.4	301.5 ± 24.4	302.2 ± 27.9	290.2 ± 32.2	277.3 ± 24.6	243.3 ± 20.2

a)



b)

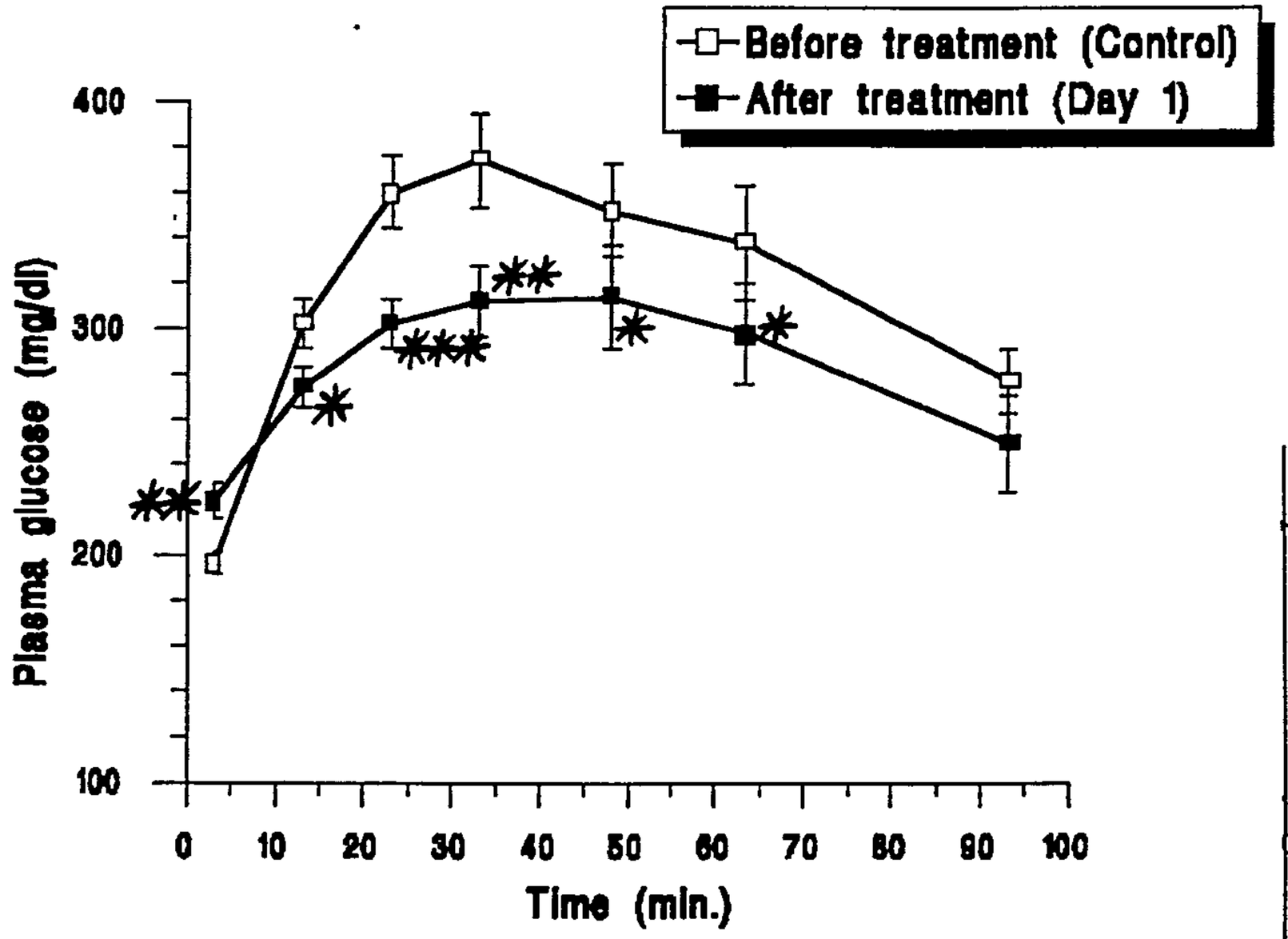


Figure 5.10: Effect of orally administered *a) 5,25-stigmastadienol* (10ml(4.6mg)/kg body weight) and *b) β-sitosterol* (10ml(3.1mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; compounds tested were given 30 min before glucose. Values of plasma glucose are mean ± SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control, by Student's paired t-tests.

Table 5.8: Effect of orally administered 5,25-stigmastadienol (M2ItC; 10ml(4.6mg)/kg body weight) and β-sitosterol (10ml(3.1mg)/kg body weight) on plasma insulin in response to a glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose challenge was given at time 0; compounds tested were given 30 min. before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 as compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
5,25-stigmastadienol							
Control Day (n=6)	237.0 ± 32.2	330.0 ± 32.9	337.5 ± 10.8	323.7 ± 24.3	335.2 ± 36.9	314.3 ± 36.8	322.3 ± 42.0
Day 1 (n=6)	257.8 ± 44.7	345.5 ± 30.4	339.7 ± 38.8	294.3 ± 17.9	302.5 ± 34.5	245.0 ± 38.8	268.7 ± 37.4
β-sitosterol							
Control Day (n=6)	212.8 ± 28.0	274.5 ± 23.5	284.3 ± 16.5	273.0 ± 18.0	258.2 ± 47.2	250.3 ± 24.4	284.5 ± 30.3
Day 1 (n=6)	242.8 ± 34.6	290.3 ± 40.9	262.0 ± 47.5	223.2 ± 41.9	278.5 ± 46.7	263.0 ± 30.3	269.7 ± 25.8

5.6 Fractionation of extract M2C

5.6.1 Preparative thin layer chromatography

Apart from extract M2I, extract M2C also improved oral glucose tolerance in n0 STZ NIDDM rats (Section 5.3). Thus further fractionation on extract M2C was carried out in order to isolate the main component. From the TLC zone profile (Fig. 5.2), the main component in M2C is the red spot with R_f 0.67.

5.6.1(a) Materials and method

The main component (red spot with R_f 0.67) was isolated by preparative thin layer chromatography using silica gel 60 F_{254} plate (1mm in thickness), with mobile phase of $CHCl_3$: EtOAc = 1:2. Using this system, the compound had a R_f value of 0.23. Repeated PTLC procedures were carried out until a pure compound was obtained.

5.6.1(b) Results

The compound of interest (M2Ciii), 8mg, was isolated from fraction M2C by PTLC.

5.6.2 Identification of compound M2Ciii

5.6.2(a) Method

^1H NMR spectra (400MHz) and ^{13}C NMR spectra (100MHz) were obtained. In order to find out the molecular weight, electron impact and high resolution fast atom bombardment mass spectra were also obtained.

5.6.2(b) Results and discussion

All spectral data for compound M2Ciii are included in Appendix 12. From the ^1H NMR spectrum, the absence of signals in the region of 7-9ppm indicates that it is not an aromatic compound. The complexity of signals around 0.8-2ppm was very similar to that of 5,25-stigmastadienol or stigmasterol, suggesting that it may be a steroidal compound. The proton signal at 9.7ppm means that the compound has an aldehyde group. The signal at 207.9ppm in the ^{13}C NMR spectrum again indicates the presence of an aldehyde. The ^{13}C NMR spectrum showed the presence of 30 carbon signals in the molecule. The DEPT spectrum exhibited 7 methyls, 7 methylene and 10 methine signals, while the remaining 6 signals were due to the quaternary carbon atoms.

On the basis of all the NMR spectral data, the compound is likely to be a tetracyclic triterpenoid, with an aldehyde group. From an extensive literature search on all compounds previously isolated from *Momordica charantia*, one possible identity is momordicine I (Fig. 5.11), which was previously isolated from the leaves and vine by Yasuda *et al.* (1984). This paper reported only partial NMR (^{1}H and ^{13}C) spectral data for momordicine I and the compound was dissolved in deuterated pyridine rather than $CDCl_3$ (in our case).

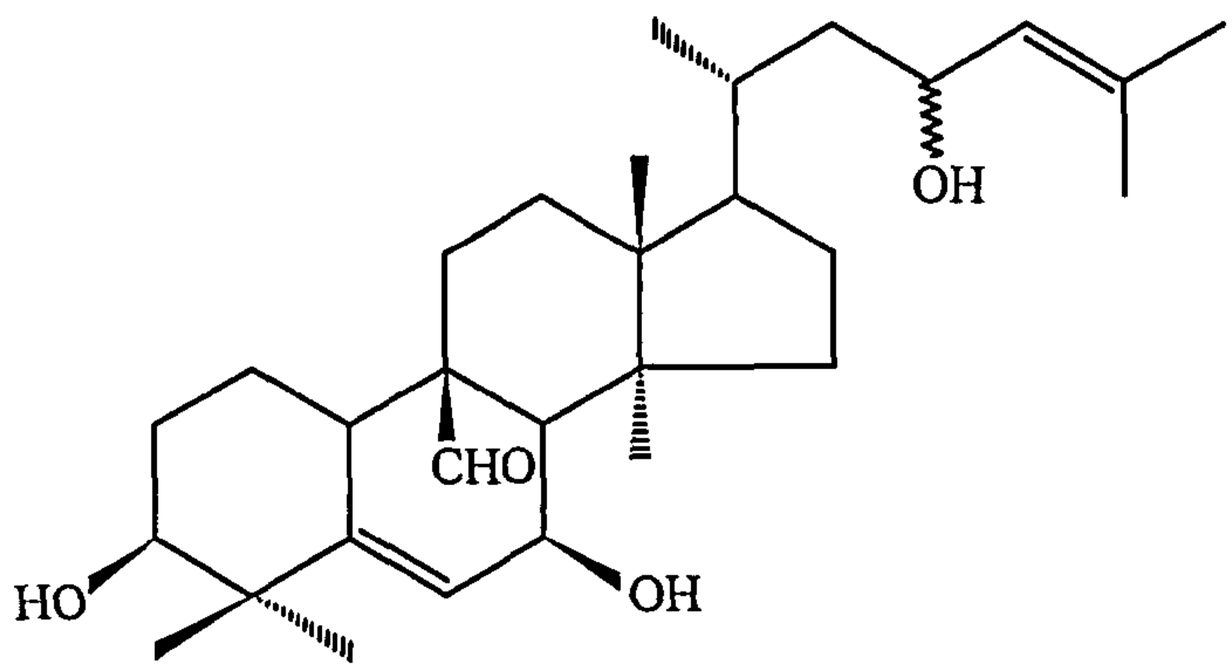
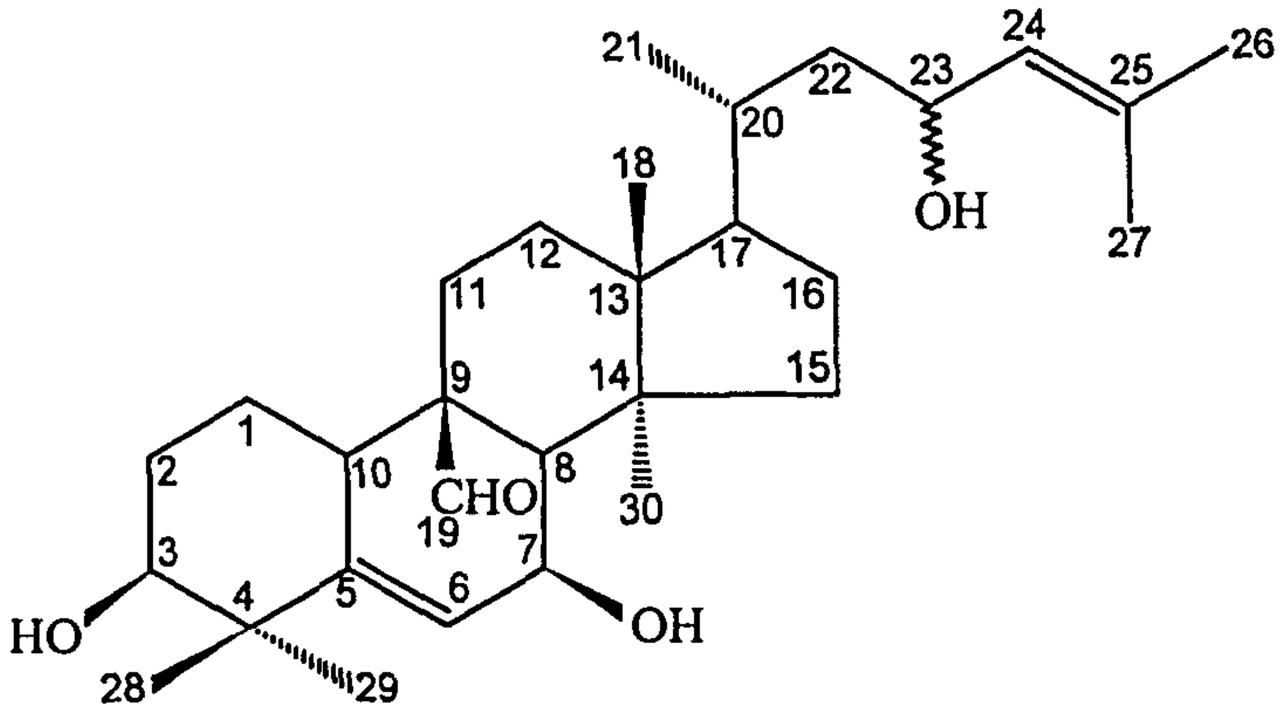


Figure 5.11: Structure of momordicine I

Thus a reference sample of momordicine I (5mg) was obtained from the corresponding author of the paper, Dr Hikaru Okabe, Faculty of Pharmaceutical Sciences, Fukuoka University, Japan. The ^{1}H and ^{13}C NMR spectra of reference momordicine I (Appendix 13) were run at King's College under the same conditions (i.e. dissolved in solvent $CDCl_3$) as our compound M2Ciii. Due to instrumental problem, the ^{13}C spectrum for reference momordicine I only covered 0-180ppm, thus the signal corresponding to the aldehyde group was not seen on the spectrum. However, from the results, the ^{1}H spectra for both reference compound and M2Ciii are virtually identical. In both cases, the ^{1}H NMR spectra showed the presence of an aldehyde group at δ9.72 (1H, singlet, **C_{19}-H**). Although the expected splitting patterns were not always observed, the following tentative proton assignments were made. We observed 3 signals in δ 3.57-4.49 region, due to protons on carbons with a hydroxy group, i.e. **C_3-H**, **C_7-H**, and **C_{23}-H**. The signal δ4.46 (multiplet) was assigned to **C_{23}-H**, a proton being close to a double bond and coupled to protons on C-22 and C-24. The signal δ3.97 (doublet; $J = 5.3$ Hz) probably corresponds to **C_7-H**,

though a double doublet splitting pattern is expected. The remaining signal δ3.57 possibly corresponds to **C_3-H**, similar to that in stigmasterol and 5,25-stigmastadienol. We again observed 3 signals in δ 5.18-5.90 region, of which the peaks at δ5.19 and δ5.89 were doublets. These may correspond to protons on C-6 and C-24 since both of which would give doublets due to coupling with protons on C-7 and C-23 respectively. Comparing to stigmasterol where the C_6-H signal was at δ5.35, the **C_6-H** signal of momordicine I will be predicted to be at δ5.19 (J = 8.5), therefore the signal at δ5.89 (J = 4.2) is assigned to **C_{24}-H**. ^{13}C assignments for compound M2Ciii and reference momordicine I (Table 5.9) were made by comparison with literature values for closely related cucurbitane triterpenoids (Fatope *et al.*, 1990). By comparing both ^{13}C NMR spectra (Table 5.9), it was shown that M2Ciii contained mainly momordicine I, though with some possible impurities present as extra carbon peaks were seen. Despite some minor differences observed in the double bond region between the two ^{13}C spectra, both ^{1}H spectra matched well in this region. The fast atom bombardment mass spectrum also confirmed the molecular weight of the compound as 472 (495-23(Na)). Thus we confirmed that the compound M2Ciii is mainly momordicine I.

Table 5.9: ^{13}C NMR ($CDCl_3$) spectral data for compound M2Ciii



Momordicine I

Carbon no.	ppm (M2Ciii)	ppm (reference compound*)
C-1	21.2	21.2
C-2	29.1	29.1
C-3	76.1	76.1
C-4	41.4	41.4
C-5	145.5	N/A
C-6	123.9	123.9
C-7	65.9	65.9
C-8	50.8	50.8
C-9	49.9	49.9
C-10	36.6	36.6
C-11	23.6	23.5
C-12	28.4	28.4
C-13	45.4	45.4
C-14	47.6	47.4
C-15	34.6	34.6
C-16	29.0	29.0
C-17	47.5	47.6
C-18	14.9	14.9
C-19	207.9	N/A
C-20	32.6	32.6
C-21	18.8	18.8
C-22	44.4	44.4
C-23	66.3	66.3
C-24	128.9	128.9
C-25	134.0	N/A
C-26	25.8	25.7
C-27	18.2[a]	18.1[a]
C-28	25.4	25.4
C-29	27.2	27.2
C-30	17.9[a]	17.9[a]

* obtained from Dr Hikaru Okabe, Fukuoka University, Japan.

N/A - not available; [a]The assignments in the same vertical column may be reversed.

5.7 Investigation on the presence of sterols and momordicine I in different varieties of karela fruit and seed

5.7.1 Introduction

From the previous sections (5.4 and 5.6), two steroidal compounds (5,25-stigmastadienol and β-sitosterol) and a tetracyclic triterpenoid (momordicine I) were found in the hexane extract of Thai karela. Thus in this section, the presence of these three compounds, together with another common steroidal compound, stigmasterol, in different varieties of karela fruit and seed will be investigated. Two methods were used, TLC and GC-MS.

5.7.2 Materials and method

Hexane extract of different varieties of karela fruit (Thai, Kenyan, Indian (Bombay), UK grown) and Thai karela seeds were prepared for investigation. Different varieties of karela juice (100ml each) were freeze-dried and the freeze-dried powder was each refluxed with hexane solvent (200ml) for 30 min. Thai karela seeds (54 seeds ≡ 5.02g) were crushed into powder using pestle and mortar and refluxed with hexane solvent (200ml) for 30 min. All the hexane extracts obtained were evaporated to dryness using a rotary evaporator.

5.7.2(a) TLC

The entire dried hexane extracts were redissolved in 1ml hexane solvent and 5μl (≡ 0.5ml original karela juice) each were applied on the plate (RP-18 F_{254S}), with solvent system – MeOH : CH_3CN (2:1). The four reference compounds, β-sitosterol, stigmasterol, 5,25-stigmastadienol and momordicine I were each dissolved in $CHCl_3$ (5mg in 0.5ml $CHCl_3$) and 5μl each (≡50μg) were applied on the plate. After development, the zones were detected under UV shortwave and also under daylight after spraying with anisaldehyde spraying agent and subsequent heating at 100°C for 10 min.

5.7.2(b) GC-MS

In section 5.4.5(b3), a GC-MS system was derived for the separation of sterols. By

using this system, further investigations were carried out on the sterol content in different varieties of karela fruit and seed.

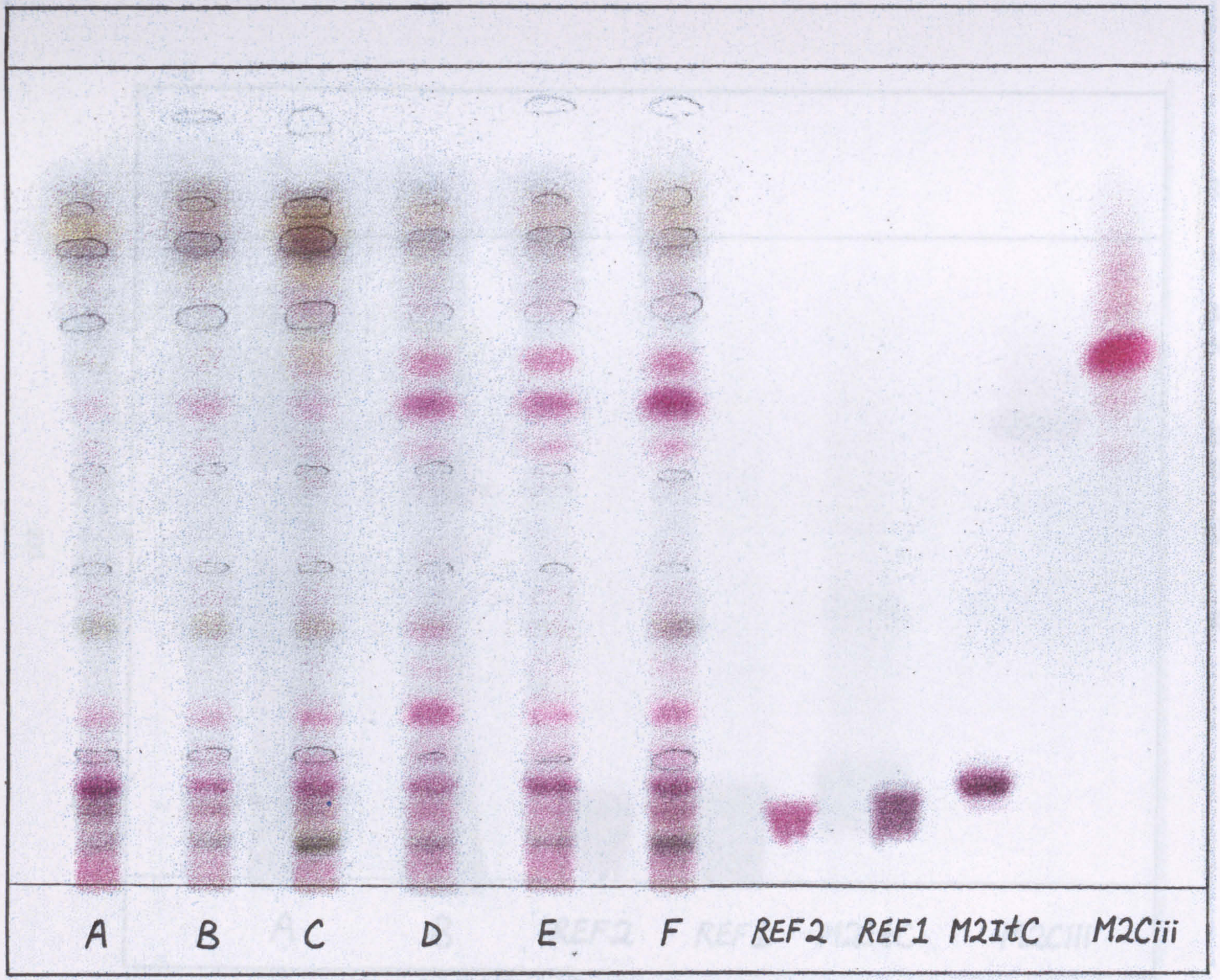
Following TLC, the remaining extract of different varieties of karela fruit and seed (approx. 1ml) were re-dried and re-dissolved in 200µl hexane prior to the analysis. The GC-MS system and the procedures were the same as before (Section 5.4.5(b3)). The reference compounds used were: β-sitosterol, 5,25-stigmastadienol and stigmasterol. A few mg (unmeasured) of the reference compounds were dissolved in 200µl $CHCl_3$ before analysis.

5.7.3 Results and discussion

The TLC zone profiles of hexane extracts of different varieties of karela fruit are shown in Fig. 5.12. From the results, the zone profiles of all hexane extracts were very similar, though minor differences (intensity of the coloured zones) were observed among different varieties and also within the same variety. By comparison with the reference compounds, 5,25-stigmastadienol was present in all varieties, whereas momordicine I was only detectable in Thai and UK karela. Due to the relatively similar R_f values and zones colour for compounds β-sitosterol and stigmasterol, it is difficult to comment on their individual presence in different varieties of karela fruit. However, the presence or absence of one or both compounds in different varieties of karela fruit was further confirmed by the GC-MS analysis.

Figure 5.13 shows the zone profile of hexane extracts of Thai karela fruit and seed. The zone profiles between the fruit and the seed are very different, a few components present in the seed were not found in the fruit (e.g. zones x and y) and vice versa. It is interesting to note that momordicine I was not present in the seed, and the compound 5,25-stigmastadienol was also likely to be absent. Again, the presence or absence of this compound as well as β-sitosterol and stigmasterol in the seed was further investigated by the GC-MS analysis.

Results obtained from the GC-MS analysis are shown in Table 5.10. From the results, Thai, Kenyan and UK grown karela fruit all contained β-sitosterol and 5,25-stigmastadienol, but not stigmasterol, and the results are consistent among different



RP-18 F_{254S} plate

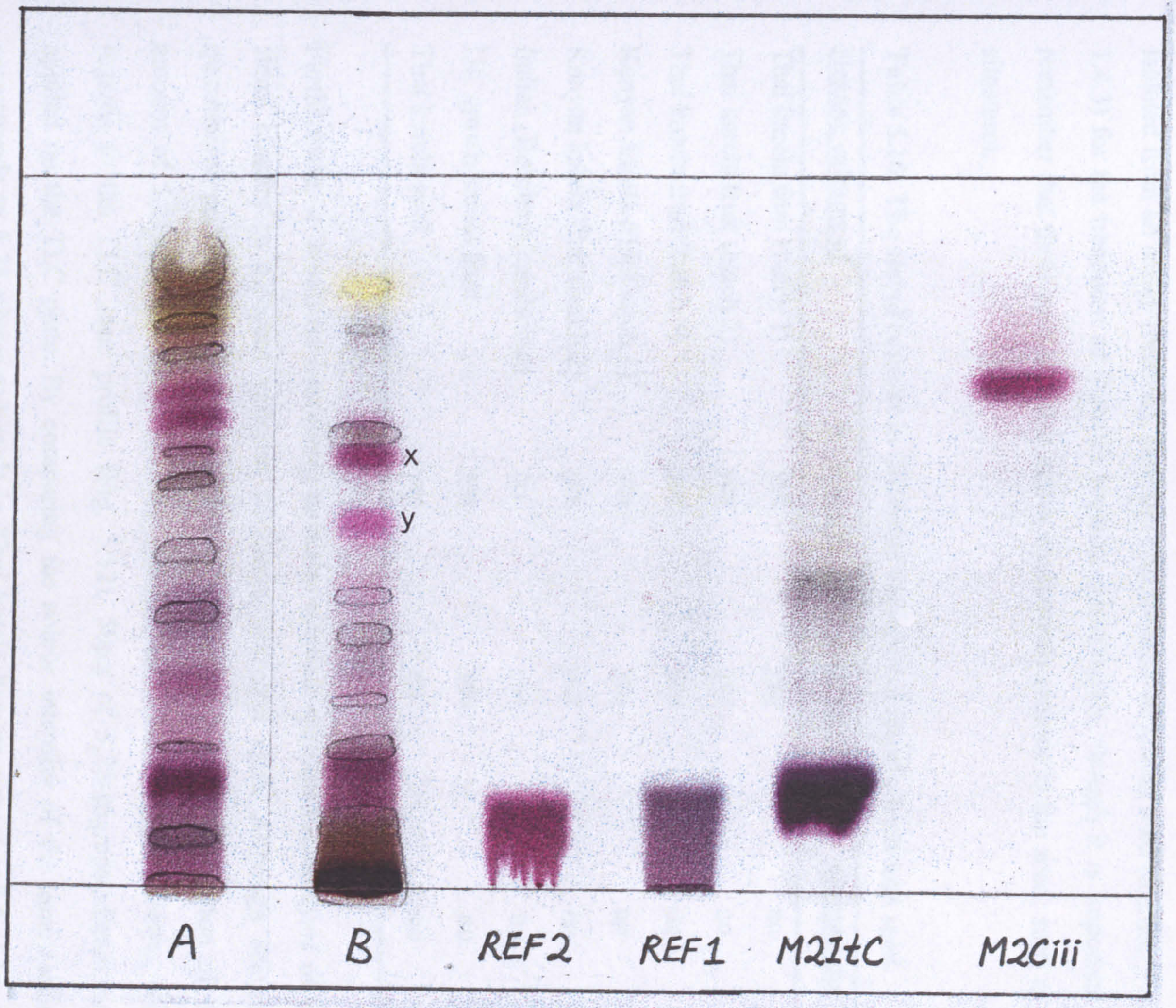
Application : 5µl

Solvent system -
MeOH : CH_3CN
2 : 1

Detection -
i) UV shortwave (254nm)
(zones marked by pencil)
ii) Anisaldehyde spraying agent

Keys: A & B = Kenyan
C = Indian (Bombay)
D = UK
E & F = Thai
REF 1 = stigmasterol
REF 2 = β-sitosterol
M2ItC = 5,25-stigma-stadienol
M2Ciii = momordicine I

Figure 5.12: TLC zone profiles of hexane extracts of different varieties of karela fruit as compared to reference compounds



RP-18 F_{254S} plate

Application : 10µl

Solvent system -
MeOH : CH_3CN
2 : 1

Detection -
i) UV shortwave (254nm)
(zones marked by pencil)
ii) Anisaldehyde spraying agent

Keys:
A = Hexane extract of Thai karela fruit
B = Hexane extract of Thai karela seed
REF 1 = stigmasterol
REF 2 = β-sitosterol
M2ItC = 5,25-stigmastadienol
M2Ciii = momordicine I

Figure 5.13: TLC zone profiles of hexane extracts of Thai karela fruit and seed as compared to reference compounds

batches of the same variety. However, the Indian karela only contained 5,25-stigmastadienol, and not the other 2 sterols. Thai karela seed, unlike the fruit, only contained β-sitosterol, and not 5,25-stigmastadienol or stigmasterol.

TLC and GC-MS results showed that both momordicine I and 5,25-stigmastadienol were absent in Thai karela seed. If these compounds are the active constituents responsible for the anti-hyperglycaemic effect of karela fruit (5,25-stigmastadienol was shown to improve oral glucose tolerance; section 5.5 and momordicine I was isolated from an active fraction), then the effectiveness of karela seed (Section 1.4.2-1.4.3) for the treatment of diabetes remains questionable, though it is important to remember that there may be other active compounds present in the seed, such as β-sitosterol.

Table 5.10: The sterol content in different varieties of karela fruit and seed

Hexane extracts of	β-sitosterol	5,25-stigmastadienol	stigmasterol
Thai karela fruit (batch 1)	yes	yes	no
Thai karela fruit (batch 2)	yes	yes	no
Thai karela fruit (batch 3)	yes	yes	no
Kenyan karela fruit (batch 1)	yes	yes	no
Kenyan karela fruit (batch 2)	yes	yes	no
Indian (Bombay) karela fruit	no	yes	no
UK grown karela fruit	yes	yes	no
Thai karela seed	yes	no	no

Furthermore, it would be interesting to make accurate quantitative analysis of the sterol content in different varieties of karela fruit and seed. Although accurate quantitative analysis was not performed in our study, a rough estimation of the amount of 5,25-stigmastadienol in Thai karela juice was made as follows. With regards to the TLC zone profile (Fig. 5.12), 50μg of 5,25-stigmastadienol was applied on the TLC plate. By comparing the colour intensity of the zone (which corresponds to 5,25-stigmastadienol) in Thai karela hexane extract (≡0.5ml karela

juice), a rough estimate of 25µg of 5,25-stigmastadienol is being present. Thus about 0.5mg of 5,25-stigmastadienol was present in 10ml of karela juice. However, the OGTT study (Section 5.5) showed that about 5mg/kg of 5,25-stigmastadienol was required to produce a effect, which is equivalent to a dose of 100ml of karela juice/kg body weight of rat. Therefore, we can conclude that 5,25-stigmastadienol is not the sole active component in karela.

5.8 Acid hydrolysis of the karela water extract

5.8.1 Introduction

In Chapter 3, both hexane and water extracts of Thai karela were shown to have anti-hyperglycaemic activity. In the present chapter, the compounds identified in the active fractions of the hexane extract are 5,25-stigmastadienol, momordicine I and β-sitosterol. There is a possibility that their corresponding glucosides may be present in the water extract and are responsible for the observed anti-hyperglycaemic activity. Since acid can hydrolyse O-glycosides to give their corresponding aglycones and sugars, acid hydrolysis of the water extract will be carried out to see if glycosides of the three steroidal/triterpenoid aglycones are present.

5.8.2 Materials and method

Sulphuric acid (20%v/v; 50ml) was added to karela water extract (50ml). The mixture was refluxed for 1 hour, then extracted with solvents hexane (3 × 50ml), followed by chloroform (3 × 50ml). The extracts were concentrated down by rotary evaporator. For comparison, the water extract, 50ml, (without undergoing acid-hydrolysis), was also extracted with hexane and $CHCl_3$.

All extracts (the crude water extract, the hexane and $CHCl_3$ extracts obtained from the water extract with or without acid-hydrolysis, the remaining aqueous mixtures) were evaporated to dryness and then redissolved in 1ml appropriate solvent before application. All extracts, together with the reference compounds, 5,25-stigmastadienol, momordicine I, β-sitosterol and stigmasterol (5mg each individually dissolved in 1ml $CHCl_3$), were all applied (10µl each) on the TLC plate (RP-18 F_{254S}), with solvent system – MeOH : CH_3CN (2:1). After development, the zones were detected under UV shortwave and also under daylight after spraying with anisaldehyde spraying agent and subsequent heating at 100°C for 10 min.

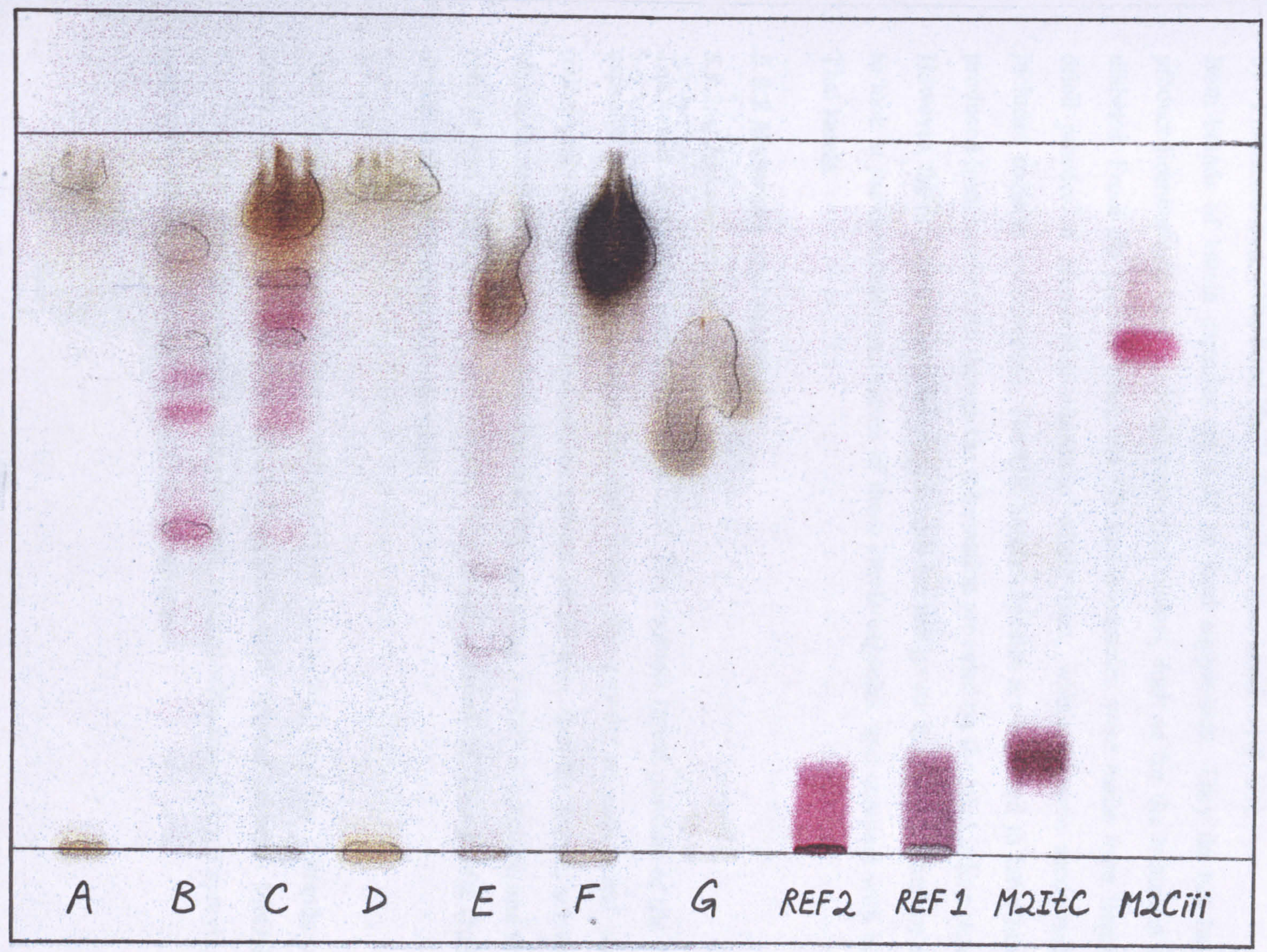
As a positive control to validate the acid-hydrolysis method, glycyrrhizic acid (a triterpene saponin from liquorice; Sigma) was acid-hydrolysed under the same conditions as above. The TLC system used was: silica gel GF_{254} plate; solvent system - ethyl acetate: ammonia, 1M: absolute ethanol (60:27:13). After development, the

zones were detected under UV shortwave and after spraying with anisaldehyde spraying agent as above.

5.8.3 Results and discussion

Examination of the hydrolysed glycyrrhizic acid showed that effective hydrolysis had occurred under these conditions, i.e. the corresponding aglycone glycyrrhetinic acid (reference compound from Sigma) was produced. This validated the experimental method.

The TLC zone profiles of karela extracts are shown in Fig. 5.14. From the results, the four reference aglycones were not found in the crude water extract (A) or any of the extracts (B-G) applied. Thus we can conclude that the compounds responsible for the anti-hyperglycaemic effect of the water extract were not the glucosides of the corresponding aglycones (5,25-stigmastadienol, momordicine I, β-sitosterol) which were present in the hexane extract. However, other possible sterols or steroids (as seen by pink zones on the TLC plate; Fig. 5.14) were observed in the non-hydrolysed extracts. However on hydrolysis, none of the reference aglycones were detected, despite the fact that the pink zones seen in the non-hydrolysed extracts were also no longer visible.



RP-18 F_{254S} plate

Application : 10µl

Solvent system -
MeOH : CH_3CN
2 : 1

Detection -
i) UV shortwave (254nm) (zones marked by pencil)
ii) Anisaldehyde spraying agent

Keys:
A = water extract
B = hexane extract
C = chloroform extract
D = aqueous layer
(B-D: without acid-hydrolysis)
E = hexane extract
F = chloroform extract
G = aqueous layer
(E-G: after acid-hydrolysis)
REF 1 = stigmasterol
REF 2 = β-sitosterol
M2ItC = 5,25-stigmastadienol
M2Ciii = momordicine I

Figure 5.14: TLC zone profiles of karela water extracts (with and without acid hydrolysis) as compared to reference compounds

5.9 Investigation on the constituents of commercial karela capsules

5.9.1 Introduction

Two different brands of commercial karela capsules were bought (Appendix 14):

a) Purchased from "The Herb Room", Santa Cruz, USA.

b) Purchased from "The Food Hall", Turnpike Lane, London N8, UK.

Both brands of karela capsules are sold as food supplement. They do not have product licences and have made no medicinal claims, such as for the treatment of diabetes. From the product label, the US karela capsules were made from freeze-dried powder of *Momordica charantia* whole fruit; whether karela seeds were included remains questionable. The UK karela capsules are believed to have been produced from karela fruit, though the information provided on the label is less clear. However, the origins of karela for both brands are not given. It would be interesting to look at the chemical constituents of these karela capsules, and compare with our Thai karela.

5.9.2 Materials and method

5.9.2(a) Hexane extract

Ten karela capsules of each brand were used. The contents (dried powder) of the 10 capsules were combined together for each brand. The powder of each brand was individually refluxed with 50ml hexane solvent for 30 min, then the hexane solvent was collected after filtration, evaporated to dryness using a rotary evaporator, and the residue was redissolved in 1ml hexane. The hexane extract of Thai karela (M2) prepared earlier (Section 5.2) was used.

5.9.2(b) TLC

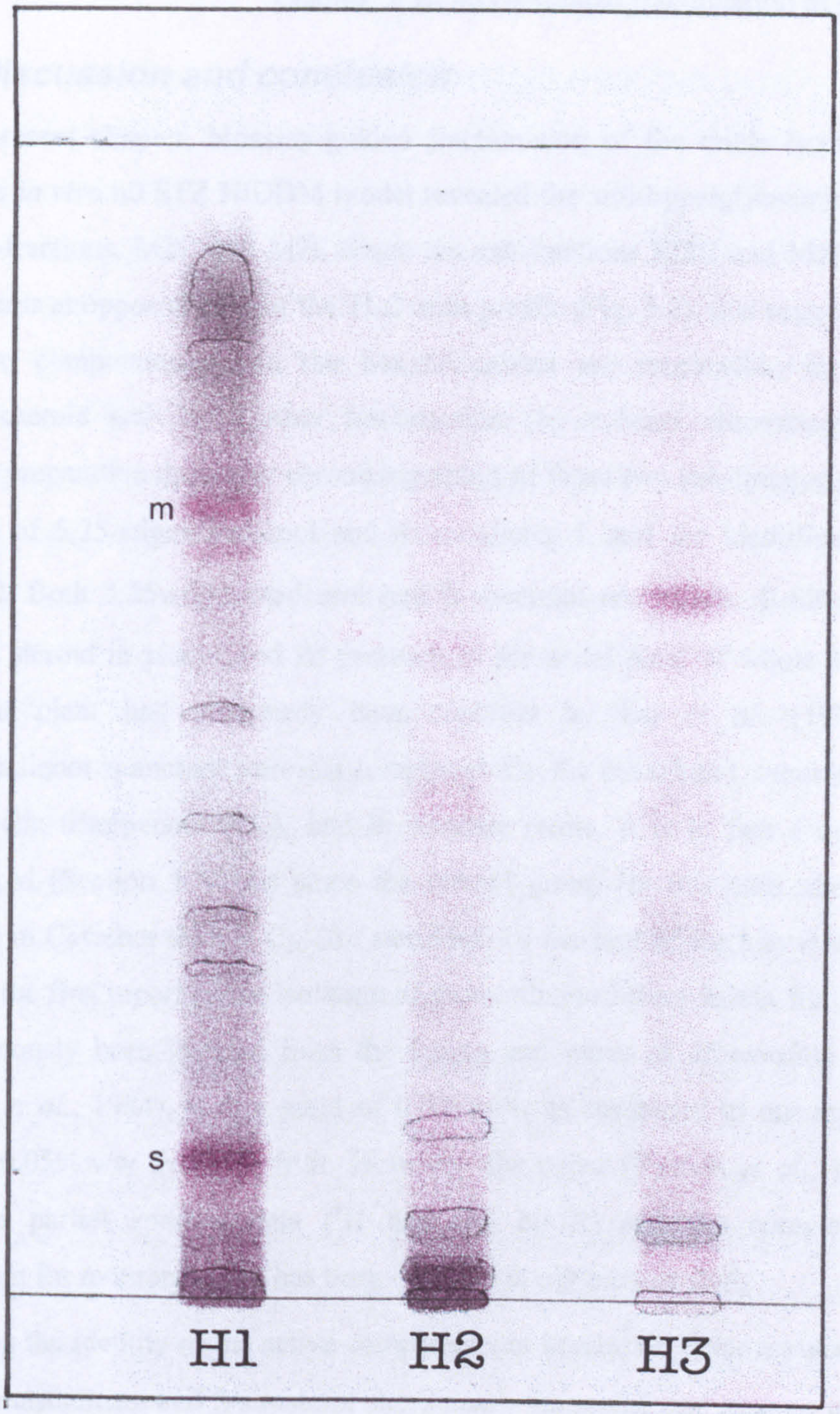
10µl of each reconstituted hexane extracts (Thai karela (M2) and the 2 brands of karela capsules) were applied on RP-18 F_{254S} plate, with solvent system - MeOH: CH_3CN (2:1). After development, the plate was sprayed with anisaldehyde spraying agent with subsequent heating at 100°C for 10 minutes.

5.9.3 Results and discussion

The total amount of karela dried powder in 10 of the US and UK karela capsules were 2.194g and 2.053g respectively. The dried powder of UK karela capsules was fairly coarse and brown in colour; whereas the dried powder of US karela capsules was very fine and yellow-brown in colour.

The TLC zone profile is shown in Fig. 5.15. The zone profiles for the hexane extracts between the US and UK karela capsules were very different, and they were also different from that of our Thai karela. It is amusing to observe that the two compounds (5,25-stigmastadienol and momordicine I) isolated from the active fractions of Thai karela extract were not detected in both karela capsules hexane extracts, despite the fact that one of the brands (US karela capsules) claims to maintain the biologically active constituents. Although both brands of karela capsules are sold as food supplement, if the public is to use them as anti-diabetic remedy, then their effectiveness remains questionable. In addition, based on information in Fig. 5.1, the freeze-dried powder of 10 capsules ≡ about 70ml karela juice. Thus each capsule is only equivalent to about 7ml of karela juice. According to the recommended dosages, one capsule (UK brand) or 1-3 capsules (US brand) should be taken daily. In both cases, the doses taken are very low (≡ about 7-21ml karela juice per day).

Our results showed that the chemical constituents of commercially available karela capsules can vary considerably between brands. So far, the author has not seen any commercial karela products in market which are sold specifically for the treatment of diabetes. However, if this is the case in the future, then the quality control and standardisation of active constituents will undoubtedly be the main issue.



RP-18 F_{254S} plate; Application : 10µl

Solvent system - MeOH : CH_3CN (2:1)

Detection -
i) UV shortwave 254nm (zones marked by pencil)
ii) Anisaldehyde spraying agent

Keys: H1 - Thai karela (M2); H2 - UK karela capsules; H3 - US karela capsules
m = momordicine I; s = 5,25-stigmastadienol

Figure 5.15: TLC zone profiles of hexane extracts of commercial karela capsules

5.10 Discussion and conclusion

In the present chapter, bioassay-guided fractionation of the crude hexane extract using the *in vivo* n0 STZ NIDDM model revealed the anti-hyperglycaemic effects of two sub-fractions, M2C and M2I. Since the sub-fractions M2C and M2I contained components at opposite ends of the TLC zone profile (Fig. 5.2), this suggested that at least two compounds within the hexane extract are responsible for the anti-hyperglycaemic activity. Further fractionation (by column chromatography and repeated preparative thin layer chromatography) of these two sub-fractions led to the isolation of 5,25-stigmastadienol and momordicine I, and the identification of β-sitosterol. Both 5,25-stigmastadienol and β-sitosterol are sterols. β-Sitosterol is a common steroid in plants, and its presence in the aerial parts of whole *Momordica charantia* plant had previously been reported by Lal *et al.* (1990). 5,25-Stigmastadienol is another steroidal compound. On the other hand, momordicine I is a tetracyclic triterpenoid (C_{30}), and in accurate terms, it is in fact a cucurbitacin triterpenoid (Section 1.3.1(c)) since the methyl group (in this case, the aldehyde group) is in C_9 rather than in C_{10} (for steroids). To the best of our knowledge, this is possibly the first report of the isolation of momordicine I from karela fruit, though it had previously been isolated from the leaves and vines of *Momordica charantia* (Yasuda *et al.*, 1984), with a yield of 0.07%w/w as compared to our approximate yield of 0.05%w/w from the fruit. However, the paper (Yasuda *et al.*, 1984) only published partial spectral data (^1H and ^{13}C NMR) and the complete carbon assignment for momordicine I has been reported in our present study.

Regarding the identity of the active components in karela, we demonstrated that both 5,25-stigmastadienol and β-sitosterol significantly improved oral glucose tolerance in the n0 STZ NIDDM model. In addition, momordicine I is the main component in the active sub-fraction M2C, though its anti-hyperglycaemic activity is still to be investigated. One reason for not testing momordicine I on oral glucose tolerance was that the total amount isolated (about 10mg) was not enough for the *in vivo* testing. Secondly, since momordicine I is a triterpenoid, it was not regarded by Lipha as being of sufficient interest for further investigation of its anti-diabetic activity, considering the resources that would be needed for this.

The isolation of both 5,25-stigmastadienol and β-sitosterol from an active fraction of karela is in agreement with the isolation of the compound charantin in the early sixties. Charantin (Fig. 1.19), a 1:1 mixture of β-sitosterol and 5,25-stigmastadienol glucosides was isolated from *Momordica charantia* fruit by Lotlikar and Rajarama Rao (1960/61). Charantin was claimed to be the active constituent in *Momordica charantia* since its oral or intravenous administration to normal or alloxan diabetic rabbits resulted in a significant fall in plasma glucose (Lotlikar and Rajarama Rao, 1966). It is interesting that in our study, β-sitosterol and 5,25-stigmastadienol (the aglycones of charantin) were found to be the main components in one of the active sub-fraction (M2I) of crude hexane extract and both compounds were shown to be active in OGTT. Our results in Section 5.8 have demonstrated that the water extract did not contain the glucosides of the corresponding aglycones found in the hexane extract, suggesting that other active compounds are present in the water extract. These need to be investigated further.

Earlier in Section 1.4.3, we commented on the high dosages of charantin used in the studies by Lotlikar and Rajarama Rao (1960/61; 1966). The dosages of charantin used in Lotlikar and Rajarama Rao's studies were 10-50mg/kg, whereas in our present study, only about 5mg/kg (5,25-stigmastadienol) and 3mg/kg (β-sitosterol) were used. Since smaller dosages were needed in our study, there is in fact a stronger evidence for the importance of 5,25-stigmastadienol and β-sitosterol in karela's activity. Nevertheless, from the previous discussion in Section 5.7.3, it appears that the dose of 5,25-stigmastadienol needed is much higher than an effective dose of the juice, suggesting that 5,25-stigmastadienol is not the sole active constituent in karela. The same would apply for β-sitosterol.

In summary, evidence arising from this bioassay-guided fractionation that there is more than one active component in karela is as follows:

i) The hexane and water extracts were both active, indicating that at least two compounds (one non-polar and one very polar) were present. In addition, the corresponding glycosides of those aglycones found in the hexane extract were not detected in the water extract, suggesting that other active compounds were present

in the water extract.

ii) The hexane extract itself gave 2 well separated active fractions (M2C and M2I).

iii) The doses of isolated compounds needed for an effect were much greater than doses present in the crude extract, suggesting the synergistic or additive effect of different active components in karela. For example, about 5mg/kg of 5,25-stigmastadienol was required to show a positive effect in OGTT, but this is about 3-4 times greater than its actual amount in the crude hexane extract.

Our results showed that 5,25-stigmastadienol, β-sitosterol and possibly momordicine I are all involved in the anti-hyperglycaemic activity of karela. However, variation in the presence of these compounds in different varieties of karela fruit and seed was observed (Section 5.7). This may account for the differences in potency in the anti-hyperglycaemic activity among different varieties of karela, and also differences between karela fruit and seed as anti-diabetic agent. These compounds were not detected in 2 commercial samples of freeze-dried karela (Section 5.9).

The mode of action of the two active compounds, 5,25-stigmastadienol and β-sitosterol, is still to be investigated. Our results showed that both compounds did not stimulate phase I insulin secretion. The other possible modes of action, such as their effects on the gut (intestinal glucose uptake inhibitory effect), or on the liver (hepatic insulinomimetic or insulin sensitising activity), require further investigation.

Chapter 6:

General discussion and conclusion

Chapter 6: General discussion and conclusion

6.1 General discussion

An extensive literature review on *Momordica charantia* L. (Section 1.4) showed evidence for its anti-diabetic activity in humans, animals and various *in vitro* models. However, the information on the identity of the active constituent(s) is less clear. Although a number of hypoglycaemic principles has previously been proposed, none of these appears to totally satisfy the enquiry. Thus there was clearly a need for further study on the active hypoglycaemic principles of *Momordica charantia*, in which particular attention is given to the dose and route of administration.

The aim of the present study was to use a systematic, relevant approach to isolate and identify orally active anti-diabetic phytochemicals in the unripe fruit of *Momordica charantia*, and to elucidate the mode of action.

The present study has highlighted a few interesting findings which are believed to provide a useful scientific contribution to the research on *Momordica charantia* fruit as an anti-diabetic remedy. Some of the findings are novel, whereas others support previously published data through the use of a different approach. In this Chapter, an attempt is made to summarise the important findings of this study. Suggestions for future work are given in section 6.2.

At the beginning of our laboratory investigations (Chapter 2), we reported that karela fruit from different geographical origins (Africa (Kenya and Nigeria), Bangladesh, India (Bombay and Jaipur), Thailand and UK) were different in size, shape, colour and even had different chemical constituent profiles. Furthermore, our investigation (Chapter 3) showed differences in potency in the biological activities between two varieties of karela fruit (Thai and Kenyan). Previous studies on *Momordica charantia* fruit have seldom specified the geographical source or cultivar of karela used, which may partly account for differences among researchers' results. In Chapter 5, we observed that the presence of compounds associated with the activity was somewhat variable among different varieties of karela. A comparison of different varieties of karela has not previously been reported, thus our study highlighted the importance

of recording the origin of karela fruit used for research studies.

In the majority of previous studies, compounds were isolated from karela fruit with no apparent rationale and then tested for their anti-diabetic activities. In addition, the doses employed were not compared to the concentration of the substances in the original crude extracts. Thus a clear indication of the relevance of the single compound to the activity of the whole fruit was not given. Besides, tests were not always performed with the oral route of administration, sometimes intravenous injections were used, e.g. p-insulin (Khanna *et al.*, 1981). Unlike previous studies, our study attempted to use a systematic, relevant approach to isolate the orally active constituent(s) in karela. Our aim was also to pay attention to the concentration of these constituents in the whole karela juice and further fractions while performing the tests. Here, an *in vivo* bioassay-guided fractionation, using n0 STZ NIDDM model rats (Chapter 3), was employed to identify the active component(s) in karela fruit. Despite previous reports on the hypoglycaemic effect of administration of karela juice/extracts in diabetic animals (Sharma *et al.*, 1960; Higashino *et al.*, 1992; Ali *et al.*, 1993a), our study showed that both Thai and Kenyan karela did not produce a statistically significant effect on basal glycaemia on NIDDM model rats. In contrast, from the oral glucose tolerance tests, we confirmed that both varieties of karela exhibited anti-hyperglycaemic activities, though Thai karela had a more pronounced effect. Thus our study focused on Thai karela.

From sequential soxhlet extraction, the most polar (water extract) and the most non-polar (hexane extract) of karela were shown to significantly improve oral glucose tolerance. Bioassay-guided fractionation of the hexane extract resulted in the isolation or identification of three compounds: 5,25-stigmastadienol (approx. 0.15%w/w yield), momordicine I (approx. 0.05%w/w yield) and β-sitosterol. This is the first report of momordicine I in *Momordica charantia* fruit, though its presence in the leaves and vines of *Momordica charantia* was previously reported by Yasuda *et al.* (1984). We observed that both compounds, 5,25-stigmastadienol (4.6mg/kg) and β-sitosterol (3.1mg/kg), significantly improved oral glucose tolerance in n0 STZ diabetic rats. Although it was disappointing not to discover novel anti-diabetic compounds, these results did support the earlier proposal (Lotlikar and Rajarama

Rao, 1960/61; 1966) that charantin (a 1:1 mixture of 5,25-stigmastadienol and β-sitosterol glucosides) is an active constituent of karela fruit. It is interesting that a much lower dose of 5,25-stigmastadienol (4.6mg/kg) and β-sitosterol (3.1mg/kg) was used in our study as opposed to the relatively high dose of charantin used (10-50mg/kg) in the other study. Charantin is often stated to be "the active" constituent of karela. It was the high dose administered by Lotlikar and Rajarama Rao (1960/61; 1966) which led us to question the role of these 2 compounds in the activity of karela. Our study strongly suggested that charantin is not the sole active constituent in karela. Firstly, another sub-fraction extract (M2C) where momordicine I was isolated, as well as the water extract (which did not contain charantin) were also active in improving oral glucose tolerance. Secondly, the effective dose of 5,25-stigmastadienol used in our study was already 10 times more than the actual amount in the juice. However, it is important to note that the doses of 5,25-stigmastadienol and β-sitosterol used were much lower than that of metformin (200mg/kg), a positive control in our study. The steroidal (lipophilic) nature of the active compounds may be related to the traditional practice of consuming fried karela as anti-diabetic remedy. This was shown (Leatherdale *et al.*, 1981) to result in an improvement in glucose tolerance in NIDDM humans. This may be due to the fact that consumption of karela in a lipophilic matrix may aid the absorption of these sterols. The anti-hyperglycaemic activity of momordicine I is still to be investigated.

From the pharmaceutical industry (Lipha) point of view, since all three compounds (5,25-stigmastadienol, momordicine I and β-sitosterol) are of steroidal/triterpenoidal structure, and the fact that uses of high doses of steroids may result in wide range of side effects (BNF 1998), their use as lead compounds for development of new orally active anti-diabetic agents is not encouraged. However, a recent review article by Ling and Jones (1995) on dietary phytosterols (such as β-sitosterol and stigmasterol) concluded that no obvious side effects associated with phytosterols have been observed, except in individuals with phytosterolemia, an inherited lipid disorder. It is known that phytosterols are generally poorly absorbed from the intestine (Ling and Jones, 1995), especially in hyperinsulinaemic NIDDM patients (Sutherland *et al.*,

1992).

The active components of the water extract of karela are still to be investigated. So far, it is encouraging to learn that the active compounds present in the water extract were not the corresponding glycosides of the aglycones (the three compounds above) present in the hexane extract (section 5.8). This suggests the presence in karela of other, possibly non-steroidal, active components which may be of greater interest for further drug development.

Phytosterols (such as β-sitosterol and stigmasterol), as the name implies, are common components of a wide range of plants. This raises the question as to whether or not all these plants possess anti-hyperglycaemic activities. The reason this is not observed may be because firstly, the amount of sterols present is insufficient to produce an effect and, secondly, because the effect of the sterols may be enhanced by the presence of other related or unrelated compounds which perhaps only occur in selected plants, e.g. *Momordica charantia*.

Our present study has also contributed useful additional information with regard to the mode of action of karela as an anti-diabetic agent. A significant finding arising from the present work was the first demonstration of an *in vivo* insulin secretagogue effect (phase I insulin stimulation) by karela (Section 3.2), which supports the previous findings of an *in vitro* insulin stimulating effect by karela (Welihinda *et al.*, 1982a,b; Ali *et al.*, 1993b; Mosihuzzaman *et al.*, 1994). Previous studies on plasma insulin have not shown any effects of karela. The stimulation of phase I insulin secretion reported here, may have been missed in earlier studies on humans (Leatherdale *et al.*, 1981) or animals (Leatherdale *et al.*, 1981; Day *et al.*, 1990; Sarkar *et al.*, 1996), due to the later initial blood sampling times (30 min or more) employed previously. In our case, the initial blood sampling time was 10 min and it was at this time when the insulin stimulation effect was seen. Though the stimulation of insulin release by karela was not clearly observed in a further study (section 3.3), this may be due to experimental error (unreliable insulin data) and large fluctuation in insulin levels. Thus further investigations should be carried out to confirm this mode of action.

Apart from the *in vivo* demonstration of phase I insulin stimulation by karela juice as mentioned earlier, the effects of karela extracts on oral, intravenous and intraperitoneal glucose tolerance were compared. To the best of our knowledge, this is possibly the first IVGTT study carried out on karela. From the results, water extract of karela only improved oral glucose tolerance, suggesting that it may work primarily on the gut, i.e. inhibiting intestinal glucose absorption. Besides, since the water extract only improved oral but not intraperitoneal glucose tolerance (IPGTT does not involve gut hormones), it could also co-stimulate secretion of gut hormones, such as GLP-1 (glucagon-like peptide) and GIP (gastric inhibitory peptide), resulting in glucose-induced insulin secretion. The water extract was also shown to stimulate insulin release (Fig. 3.9), though the effect was not as significant as in the whole juice.

The hexane extract of karela was shown to improve both oral and intraperitoneal (but not intravenous) glucose tolerance indicating that it may also have some effects on the liver, as well as inhibiting intestinal glucose absorption. In fact, alterations in hepatic enzyme levels following administration of karela have been reported in previous studies (Shibib *et al.*, 1993). The hepatic effects are more likely to be insulin sensitising rather than insulinomimetic since basal glycaemia is not affected by karela juice. In our *in vivo* studies, we observed that the effect of four days treatment was almost always better than the effect of single treatment. This cumulative effect seen may be due to the changes in hepatic enzymes.

As with the water extract, hexane extract was shown to stimulate the phase I insulin release (though the effect was not significant) in OGTT, again suggesting a possible insulin secretagogue action. Separation of the water soluble and lipophilic components by extraction may account for the diminished insulin secretagogue activity.

The inhibitory effect of hexane extract of karela on intestinal glucose absorption was further investigated, using an *in vitro* intestinal brush border membrane vesicles model (Chapter 4). The hexane extract at concentrations of 0.73µg/µl resulted in significant inhibition of glucose uptake. Our study suggested that hexane extract may

contain active compounds which act like phlorizin that inhibits Na^+-dependent/glucose transport in the brush border membrane. By estimation, the IC_{50} of the hexane extract was between 1-5mg/ml as opposed to phlorizin which has an IC_{50} of 0.05mg/ml. Regarding the value of decreasing glucose supply from the gut in the treatment of NIDDM, such compounds could be useful, either given alone or combined with other oral hypoglycaemic agents, in reducing postprandial rises of blood glucose (like the α-glucosidase inhibitor acarbose) and smooth out the 24-hour blood glucose profiles. The author believes that this is the first time where BBMV were used as an *in vitro* model for studying natural products with intestinal glucose uptake inhibitory effect (apart from phlorizin) and this could open a possibility of being used in the future as an *in vitro* model for screening anti-diabetic plants for this particular mode of action.

From our studies it appears therefore, that karela contains a number of active constituents, including 5,25-stigmastadienol and β-sitosterol which are likely to work by a range of mechanisms. This lends support to the traditional use of whole karela juice in the treatment of diabetes. However, the dose of karela juice used in our *in vivo* studies was 10ml/kg body weight of rat. For a 60kg human, the extrapolated dose will theoretically be 600ml. This may suggest that the ordinary recommended dose (50ml karela juice once or twice daily) is not effective. However, such a linear extrapolation may not be valid. Also, it must be noted that some human studies (Leatherdale *et al.*, 1981; Welihinda *et al.*, 1986) have demonstrated an effect, in which cases, the doses of 50ml and 100ml of karela juice were used respectively. Additionally, whilst 50ml karela juice may not have an acute effect, it is possible that regular consumption may lead to some biological alterations (such as alterations in hepatic enzyme levels) which improve glucose tolerance. Our observation of a cumulative effect supports this possibility.

6.2 Future work

As a result of our findings regarding *Momordica charantia* fruit with respect to its anti-diabetic activity, there are now further questions to be answered:

a) What is the active component(s) present in the water extract of karela that is responsible for the anti-hyperglycaemic activity as observed?
b) Does the hexane extract of karela possess hepatic effects?
c) What is the mode of action of the sterols in improving OGTT?
d) Are there any problems of toxicity with prolonged use of karela?

Thus the author suggests future studies to be carried out in the following areas:

6.2.1 Further investigations on the phytochemicals present in the water extract of karela

The anti-hyperglycaemic compound(s) present in the water extract of karela should be very polar. One possible identity of the active component(s) might be steroidal glycosides. However, our results have shown that 5,25-stigmastadienol, β-sitosterol and momordicine I were not detected in the acid-hydrolysed water extract (Section 5.8). Therefore the identity of the active compound(s) present in the water extract remains unknown and worthy of further investigation.

Since water extract is very polar and it is further complicated by the presence of sugars in it, the approach in fractionation of the water extract will be undoubtedly different from that for the hexane extract, i.e. different TLC systems need to be used. With our success in identifying the active constituents in the hexane extract, it would be advisable to fractionate the water extract using the same *in vivo* bioassay-guided fractionation. Any active fractions obtained will be further fractionated until individual compounds are isolated. The author suggests to remove the sugars initially from the water extract by size exclusion chromatography.

6.2.2 Further investigations on the mode of action of the hexane extract of karela

In the present research project, the effect of karela juice/extracts on insulin release (using the n0 STZ NIDDM rat model) and gastrointestinal glucose absorption (using *in vitro* BBMV model) has been investigated. However, the fact that the hexane

extract of karela improved both oral and intraperitoneal, but not intravenous, glucose tolerance suggested that the extract might have some effect on the liver. Thus further investigations should be carried out to see if the hexane extract possesses any hepatic insulin sensitising or even insulinomimetic activity. This can be carried out using isolated perfused animal liver, isolated hepatocytes or hepatoma cell lines. By using these models, the effect of the hexane extract on hepatic enzyme levels, such as glucokinase, phosphoenolpyruvate carboxykinase, fructose 2,6-bisphosphate and glycogen phosphorylase, can be measured. In addition, the effect on insulin sensitivity can also be studied in these models.

6.2.3 Further investigations on the mode of action of the water extract of karela

Our results suggest that the water extract may stimulate secretion of gut hormones, such as GLP-1 and GIP. Thus the effect of water extract on levels of these gut hormones in portal blood can be measured.

6.2.4 Further investigations on the mode of action of the sterols

Our results showed no evidence of increase in insulin secretion by the sterols (5,25-stigmastadienol and β-sitosterol). However, their effects on OGTT, IPGTT and IVGTT in n0 STZ NIDDM rats can be compared, as well as their effects on intestinal glucose uptake using the BBMV model.

6.2.5 Further investigations on the toxicity of karela juice

In order to fully evaluate the potential of karela or its components as anti-diabetic agents, it will be necessary to investigate their short and long term toxicity.

6.3 Conclusion

Our research on *Momordica charantia* fruit has strongly supported its traditional use for the treatment of diabetes mellitus. Our results have clearly shown that the juice is able to improve glucose tolerance but has little acute effect on basal glycaemia. It also appears that there is more than one active component in *M. charantia* fruit and these may exert their anti-hyperglycaemic effects by various modes of action. The active components may act synergistically or additively since separation of fractions generally necessitated an increase in dose in order to obtain a similar effect to the parent test substance. The author hopes that the active components identified and isolated from *M. charantia* fruit will be used as lead compounds for the development of new orally active anti-diabetic agents. In order to achieve this goal, careful considerations will be taken on the potency and the toxicity data of the isolated compounds.

Metformin, acarbose and guar gum are marketed anti-diabetic drugs which are all derived from natural sources. It is the wish of the author that more natural products will be used as anti-diabetics in the next millennium.

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Appendices

Appendix A: Preparation of streptozotocin (STZ) solution for injection

Buffer for dissolving STZ:

Solution 1 - citric acid (monohydrate) (Sigma) 0.1M

$C_6H_8O_7.H_2O$

Solution 2 - citric acid (trisodium salt dihydrate) (Sigma) 0.1M

$C_6H_5O_7Na_3.2H_2O$

To make up buffer: 28ml of Solution 1 + 25.5ml Solution 2, then made up to 100ml with water. The final solution should be at pH 4.5.

STZ is dissolved in the buffer prior to injection (20mg STZ/ml).

Appendix B: Calculation of dosage of pentobarbitone

Pentobarbitone (Sanofi) 6g/100ml in bottle

Dosage formula:

0.2ml saline (sodium chloride 9g in 1000ml; Meram lab.) + pentobarbitone (ml) as follows -

for female rats : (weight of rat (g) × 0.8)/1000

for male rats: weight of rat (g)/1000

Appendix 1: Effect of orally administered water (10ml/kg body weight) and metformin (200mg/kg body weight; 200mg in 10ml water) on plasma insulin in response to an oral glucose load (2g/kg) in 2h fasted n0 STZ diabetic rats. Glucose was given at time 0; water or metformin was given 30 min before glucose.

*p < 0.05, **p < 0.02, ***p < 0.01 compared with control, by Student's paired t-tests.

Treatment	Plasma insulin μU/ml (mean ± SEM)						
	0 min	10 min	20 min	30 min	45 min	60 min	90 min
Water							
Control Day (n=6)	170.0 ± 18.1	360.8 ± 87.0	293.0 ± 42.7	319.3 ± 43.1	301.0 ± 48.0	235.6 ± 45.7	172.2 ± 17.4
Day 1 (n=6)	211.3 ± 17.7	417.8 ± 66.3	379.2 ± 45.7	308.2 ± 40.1	241.7 ± 25.3	312.0 ± 53.3	285.2 ± 49.0
Day 4 (n=6)	206.2 ± 28.7	431.8 ± 68.6	358.5 ± 67.1	346.8 ± 24.5	287.0 ± 21.6	238.8 ± 33.3	201.0 ± 16.4
Metformin							
Control Day (n=5)	189.8 ± 22.2	332.4 ± 68.5	317.8 ± 50.4	273.8 ± 31.6	237.6 ± 27.8	217.2 ± 19.9	201.6 ± 24.2
Day 1 (n=5)	198.0 ± 24.4	255.0 ± 21.8	261.8 ± 29.4	227.8 ± 19.4	223.2 ± 25.5	250.6 ± 38.9	284.4 ± 56.1
Day 4 (n=5)	195.6 ± 12.3	359.0 ± 65.8	334.6 ± 62.0	286.2 ± 58.1	246.0 ± 30.9	216.0 ± 8.8	218.6 ± 21.4

Appendix 2: A table of the weight of rats used in the experiment of dose-response effect of karela juice

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with control using Student's paired t-tests.

Test substances	Weight (mean ± SEM) of rats (g) on:		
	Control Day	Test Day 1	Test Day 4
Water 10ml/kg	352.0 ± 13.1	366.0 ± 12.6 ***	369.0 ± 11.2 ***
Metformin 200mg/kg	358.3 ± 9.8	369.2 ± 10.1 ***	375.0 ± 10.4 ***
Thai karela juice			
5ml/kg	349.0 ± 13.2	361.0 ± 12.1	366.0 ± 10.5 ***
10ml/kg	355.8 ± 6.8	373.3 ± 7.4 ***	380.0 ± 7.6 ***
15ml/kg	388.0 ± 16.3	394.0 ± 14.7	402.0 ± 12.2
Kenyan karela juice			
5ml/kg	355.0 ± 7.1	367.5 ± 11.5 *	370.8 ± 10.4 ***
10ml/kg	344.2 ± 15.6	354.2 ± 15.6 ***	360.8 ± 15.2 ***
15ml/kg	345.8 ± 11.5	359.2 ± 13.6 ***	363.3 ± 14.8 ***

Appendix 3: Amount of glucose in Thai/Kenyan karela juice which is available for absorption after oral administration of the juice (at dosage of 15ml/kg) in n0 STZ diabetic rat model.

According to the HPLC analysis, Thai and Kenyan karela juice contained 8-11mM and 7-8mM of glucose respectively. Thus 10mM of glucose will be used in the following calculations.

If we assume the average weight of a n0 STZ rat used in experiment was 350g, at a dosage of 15ml/kg,

$$\text{i.e. } 15\text{ml} \times \frac{350\text{g}}{1000\text{g}} = 5.25\text{ml of karela juice would be administered to the rat.}$$

How much glucose is present in 5.25ml of karela juice?

$$\text{No. of moles} = \frac{\text{volume}}{\text{molar volume}} \times \text{Molarity}$$

$$= \frac{5.25\text{ml}}{1000\text{ml}} \times 10\text{mM}$$

$$= \frac{5.25\text{ml}}{1000\text{ml}} \times 0.01\text{M}$$

$$= 5.25 \times 10^{-5} \text{ moles}$$

$$\text{No. of moles} = \frac{\text{mass}}{\text{molar mass}} \quad \text{(N.B. molecular weight of glucose = 180.16g)}$$

$$5.25 \times 10^{-5} = \frac{\text{mass}}{180.16\text{g}}$$

Therefore, amount of glucose present = $180.16\text{g} \times 5.25 \times 10^{-5}$

$$= 9.5 \times 10^{-3}\text{ g}$$

Appendix 4: A table showing the different treatment received by the n0 STZ rats on Control and Test Days.

Treatment received on Control Day	Treatment received on Test Days
Water (10ml/kg)	Water (10ml/kg) - as negative control
0.3% v/v Tween 80 in water (10ml/kg)	0.3% v/v Tween 80 in water (10ml/kg) - as negative control
Water (10ml/kg)	Metformin (200mg/kg; 200mg in 10ml water) - as positive control
0.3% v/v Tween 80 in water (10ml/kg)	Hexane extract reconstituted in 0.3%v/v Tween 80 (10ml(2.2mg)/kg)
0.3% v/v Tween 80 in water (10ml/kg)	Chloroform extract reconstituted in 0.3% v/v Tween 80 (10ml(9.8mg)/kg)
Water (10ml/kg)	Methanol extract reconstituted in water (10ml(151.6mg)/kg)
Water (10ml/kg)	Water extract reconstituted in water (10ml(102.7mg)/kg)

Appendix 5: Protein assay for BBMV

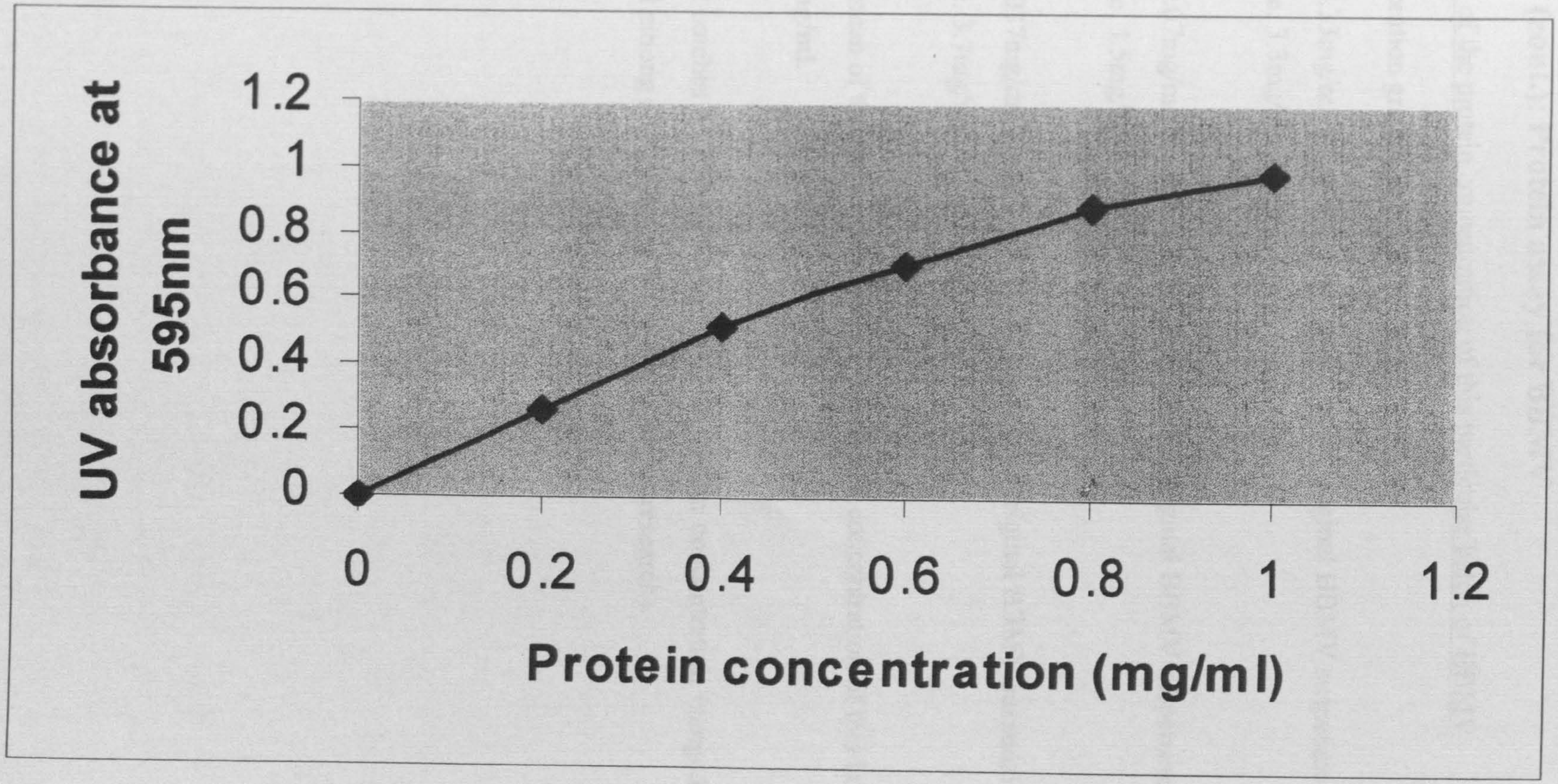
The results of the protein assay for a particular batch of BBMV are presented below.

Solutions	UV absorbance at 595nm:		
	Duplicate results	*Mean*	*Corrected values*
50µl of solution A + 950µl of dye reagent (×2)	0.587 0.593	0.590	0.0
50µl of solution B + 950µl of dye reagent (×2)	0.862 0.841	0.852	0.262
50µl of solution C + 950µl of dye reagent (×2)	1.115 1.091	1.103	0.513
50µl of solution D + 950µl of dye reagent (×2)	1.282 1.302	1.292	0.702
50µl of solution E + 950µl of dye reagent (×2)	1.464 1.476	1.470	0.880
50µl of solution F + 950µl of dye reagent (×2)	1.549 1.597	1.573	0.983
50µl of solution G (BBMV – 1 in 10 dilution) + 950µl of dye reagent (×2)	1.325 1.357	1.341	0.751
50µl of solution H (BBMV – 1 in 50 dilution) + 950µl of dye reagent (×2)	0.770 0.780	0.775	0.185
50µl of solution I (BBMV – 1 in 100 dilution) + 950µl of dye reagent (×2)	0.697 0.693	0.695	0.105

A calibration graph was drawn (see overleaf) in order to determine the protein concentration of this batch of BBMV.

cont.)

A graph of corrected absorbance against protein concentration



cont.)

Appendix 5 (cont.): Protein assay for BBMV

Determination of the protein concentration of this particular batch of BBMV

Using the calibration graph:

Solution G – 0.33mg/ml; since it is 1 in 10 dilution of original BBMV suspension,
i.e. 3.3mg/ml

Solution H – 0.07mg/ml; since it is 1 in 50 dilution of original BBMV suspension,
i.e. 3.5mg/ml

Solution I – 0.037mg/ml; since it is 1 in 100 dilution of original BBMV suspension,
i.e. 3.7mg/ml

By taking the mean of the above three values, the protein concentration of this batch of BBMV is **3.5mg/ml.**

N.B. Different batches of BBMV varied in their protein concentration (ranged from 3.5 – 8.4mg/ml among all batches of BBMV used in this research).

Appendix 6: HPLC (High Performance Liquid Chromatography) assay for sugars in karela juice/extract

The amounts of glucose and fructose in three different varieties (Indian, Kenyan and Thai) of karela juice, as well as the hexane extract of Thai karela, were quantified.

Method for HPLC assay

Glucose and fructose were well-separated from the other components in karela juice when using Apex amino (5 micron) column (20cm in length × 0.45mm internal diameter) with a mobile phase consisting of acetonitrile: water (80: 20) developed at a rate of 2.0ml/min, with a pressure of 1500psi and at room temperature. A refractive index detector (ERC-7512) was used for detection. Maltose (0.05g/ml) was used as an internal standard. A sample size of 20µl was injected into the HPLC. A set of calibration solutions was made up using varying volumes of glucose and fructose mixture (containing 5.000g of glucose and 5.000g of fructose dissolved in 100ml water) and a fixed volume (1ml) of internal standard solution, and made up to final volume of 10ml using water. The range of concentrations for glucose and fructose were 0.25 to 6.0 mg/ml.

The glucose and fructose content in different varieties of karela juice were analysed. The test solutions were prepared by adding 1ml of internal standard solution to 9ml of karela juice. In addition, the hexane extract of Thai karela was also tested for the presence of glucose and fructose. The hexane extract solution was prepared by adding 1ml of internal standard solution to the hexane extract residue (amount of residue taken was equivalent to that present in 10ml of karela juice), and the final volume adjusted to 10ml with 0.3%v/v Tween 80.

Results

From the HPLC chromatogram of test solution, fructose gave a peak at R_t (retention time) approximately 2.7min, glucose gave a peak at R_t approximately 3.1min and maltose gave a peak at R_t approximately 6.0min (Fig. A). The ratios of the peak areas of glucose or fructose to the internal standard (maltose) of the calibration solutions were plotted against the concentrations of glucose or fructose respectively. Two

straight line graphs were obtained (correlation coefficient of 0.981 for glucose; 0.975 for fructose; Fig. B).

Results are shown in Table A. Both glucose and fructose were present in all three different varieties of karela juice, with a higher concentration of glucose than fructose in all cases. The results showed differences in glucose and fructose content among the different varieties; also with slight differences between batches within a variety. However, no glucose or fructose were detected in the hexane extract of Thai karela.

Appendix 6 (cont.): HPLC assay for sugars in karela juice/extract

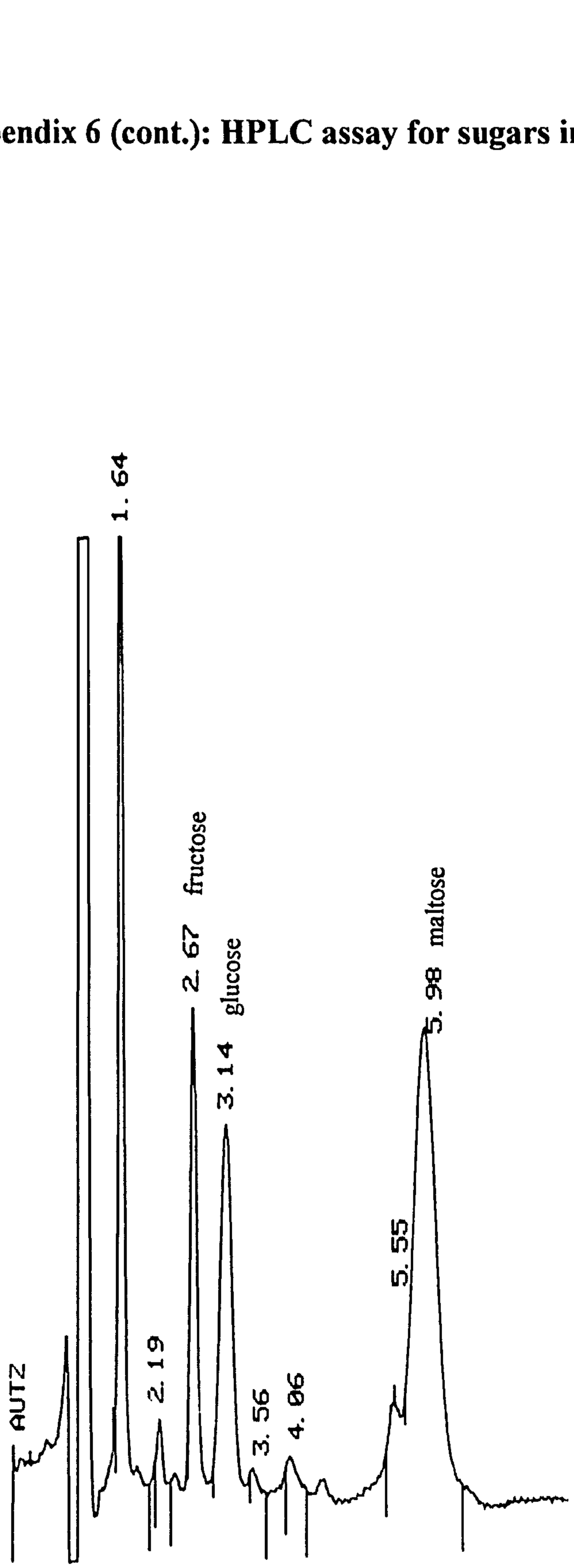


Figure A: HPLC chromatogram of Thai karela juice showing separation and retention times of sugars.

Stationary phase: Apex amino (5µ), packed in stainless steel column (20cm × 0.45mm i.d.); Mobile phase: acetonitrile: water (80:20); Flow rate: 2ml/min; Detector: refractive index (ERC-7512); Temp. 25°C; Pressure 1500psi; Sample 20µl.

Appendix 6 (cont.): HPLC assay for sugars in karela juice/extract

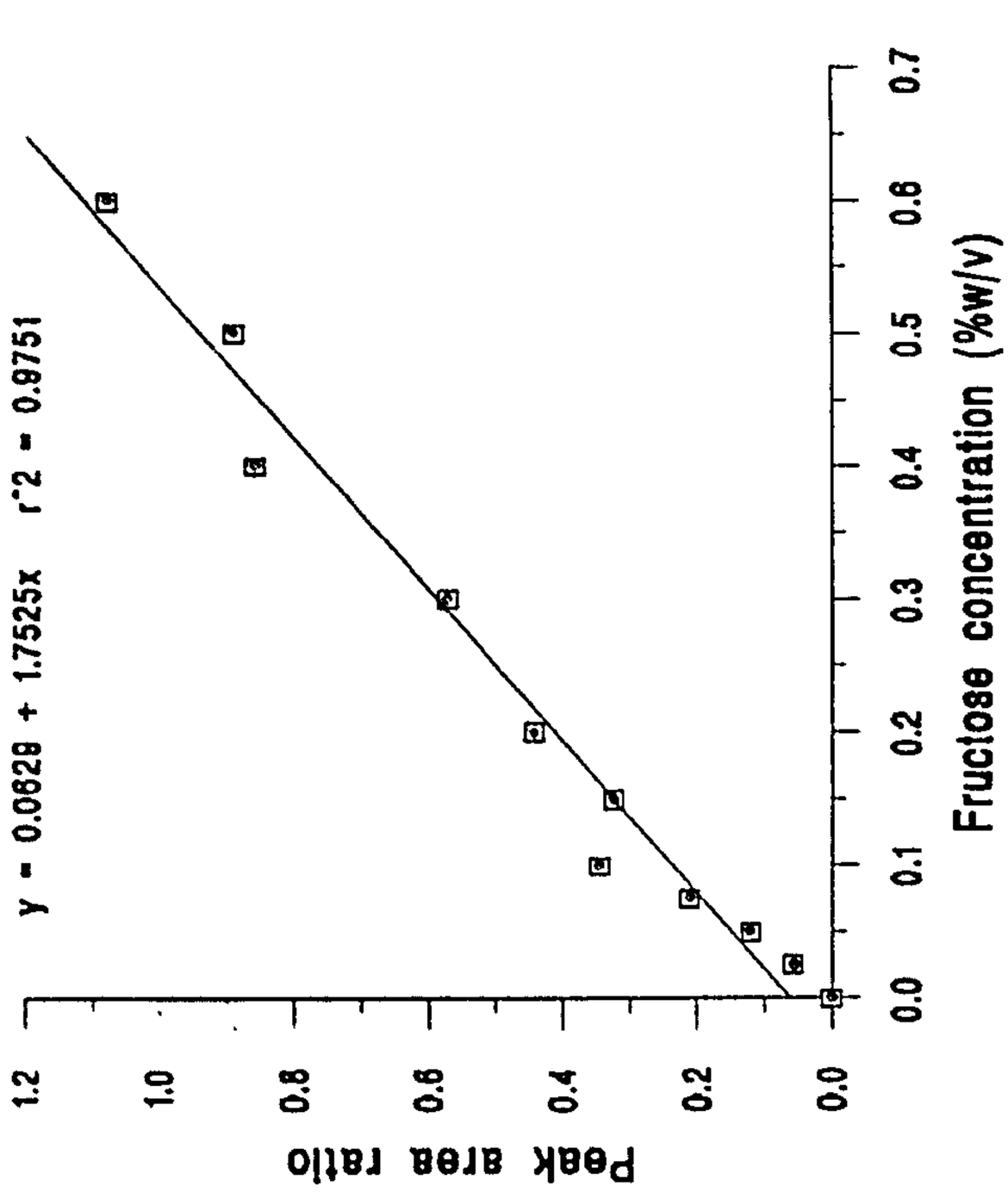


Figure A: HPLC assay of glucose and fructose

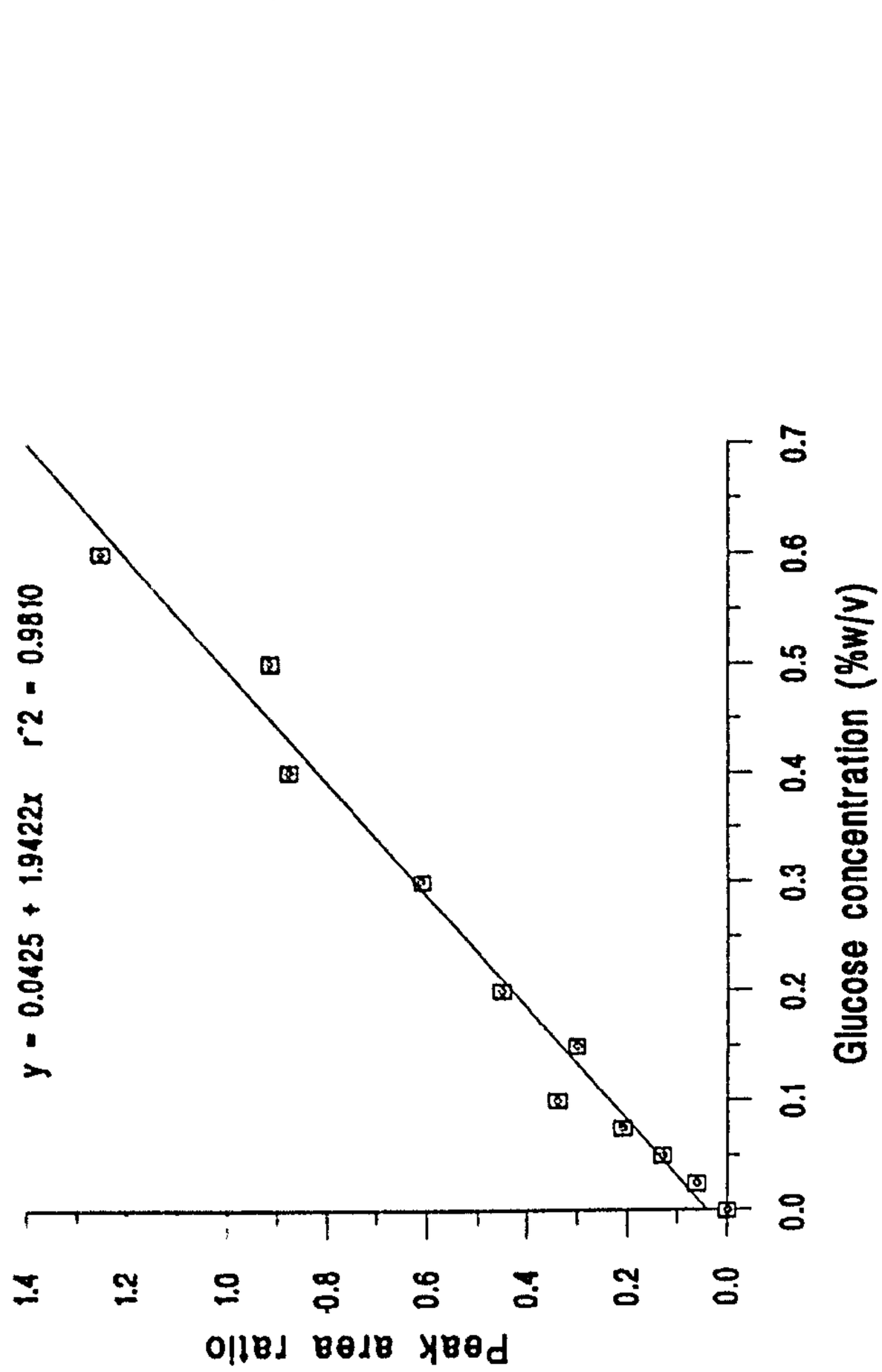


Figure B: Calibration graphs for glucose or fructose peak to that of the internal standard maltose.

N.B. Peak area ratio refers to the ratio of areas

Appendix 6 (cont.): HPLC assay for sugars in karela juice/extract

Table A: The sugar content of different varieties of karela juice/extract

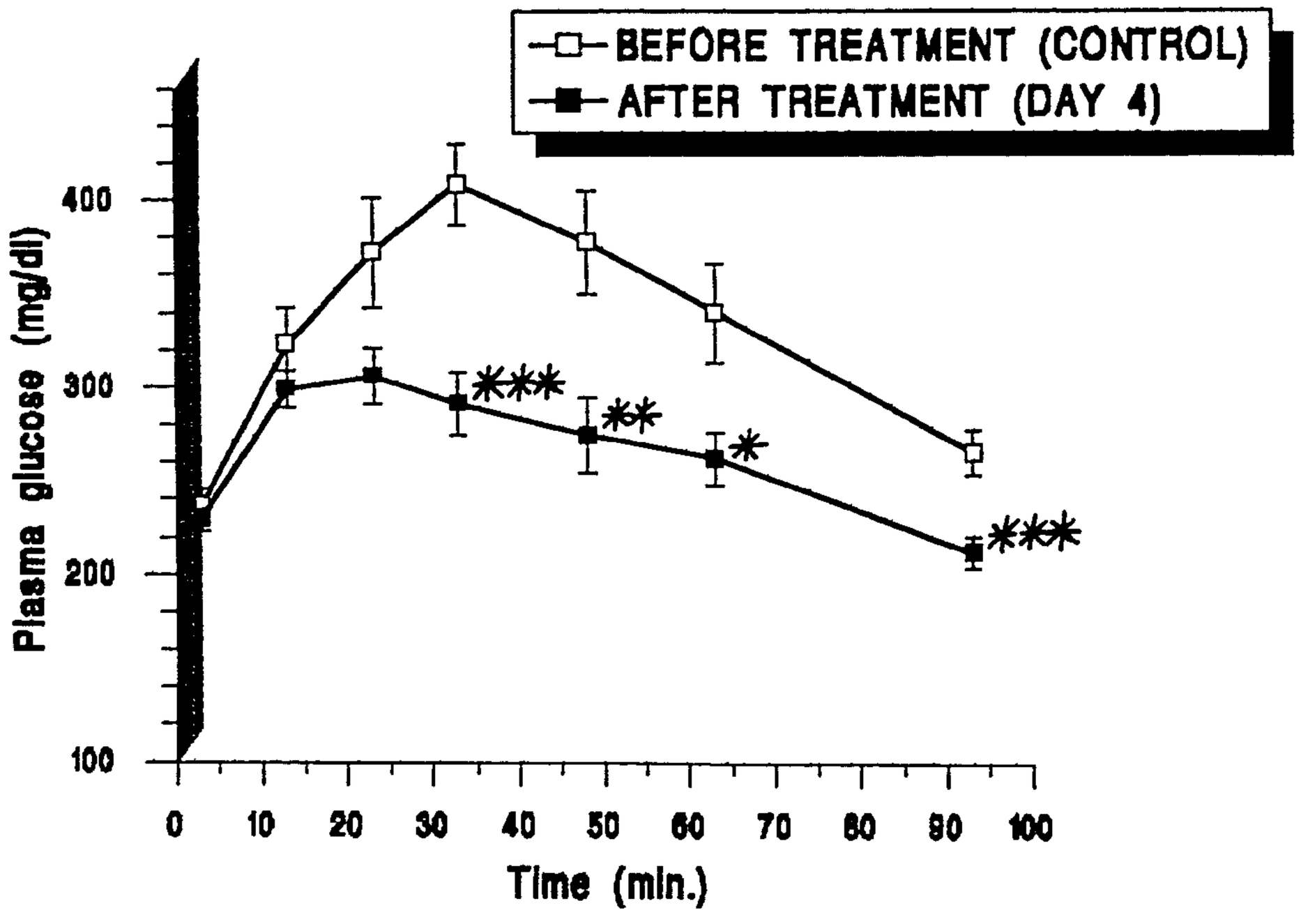
Karela juice	Glucose concentration (%w/v)	Glucose concentration (mM)	Fructose concentration (%w/v)	Fructose concentration (mM)
Indian (Jaipur)	0.151	8.39	0.021	1.14
Kenyan (Batch 1)	0.140	7.79	0.123	6.84
(Batch 2)	0.140	7.79	0.052	2.89
Thai (Batch 1)	0.150	8.36	0.125	6.94
(Batch 2)	0.163	9.05	0.148	8.24
(Batch 3)	0.185	10.26	0.126	7.00
(Batch 4)	0.159	8.85	0.108	6.02
Thai hexane extract	0.0	0.0	0.0	0.0

N.B. These calculated values are means of duplicate results.

For both glucose and fructose, 1mM = 0.018%w/v

Appendix 7: Effect of orally administered whole crude hexane extract (M2; 10ml(11.4mg)/kg body weight) on oral glucose tolerance (2g/kg) in 2h fasted n0 STZ diabetic rats (n=6). Glucose was given at time 0; karela extract was given 30 min before glucose. Values of plasma glucose are mean ± SEM.

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ compared with control, by Student's paired t-tests.



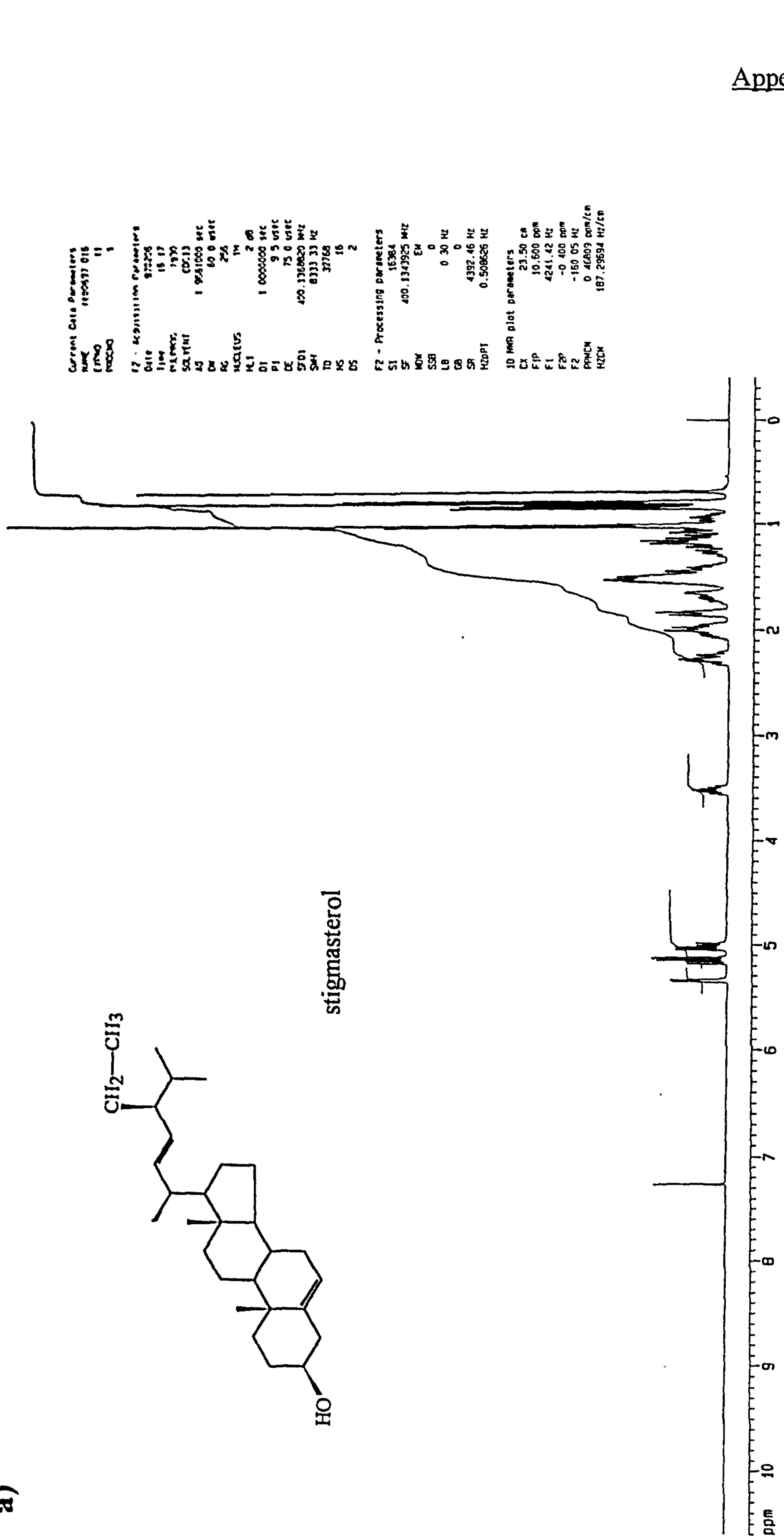
Appendix 8: NMR and MS spectra for stigmasterol

a) ^{1}H 400 MHz NMR spectrum ($CDCl_3$)

b) ^{13}C 100 MHz NMR spectrum ($CDCl_3$); full spectrum + DEPT

c) Electron impact mass spectrum

d) Fast atom bombardment mass spectrum (matrix: MNOBA + Na)



a)

stigmasterol

b)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NUCLEUS 13C
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SWH 31250 00 Hz
TD 65536
NS 512
DS 2
F2 - Processing parameters
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WDW EM
SSB 0
LB 1 00 Hz
GB 0
SR 3830 65 Hz
HZ0PT 0 953674 Hz
1D NMR plot parameters
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F1P 220.000 ppm
F1 22135 04 Hz
F2P -1.000 ppm
F2 -100 61 Hz
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77.43
77.75
122.12
129.66
138.74
141.15
20
40
60
80
100
120
140
160
180
200
ppm
STIG
stigmasterol
$CH_2—CH_3$
HO

c)

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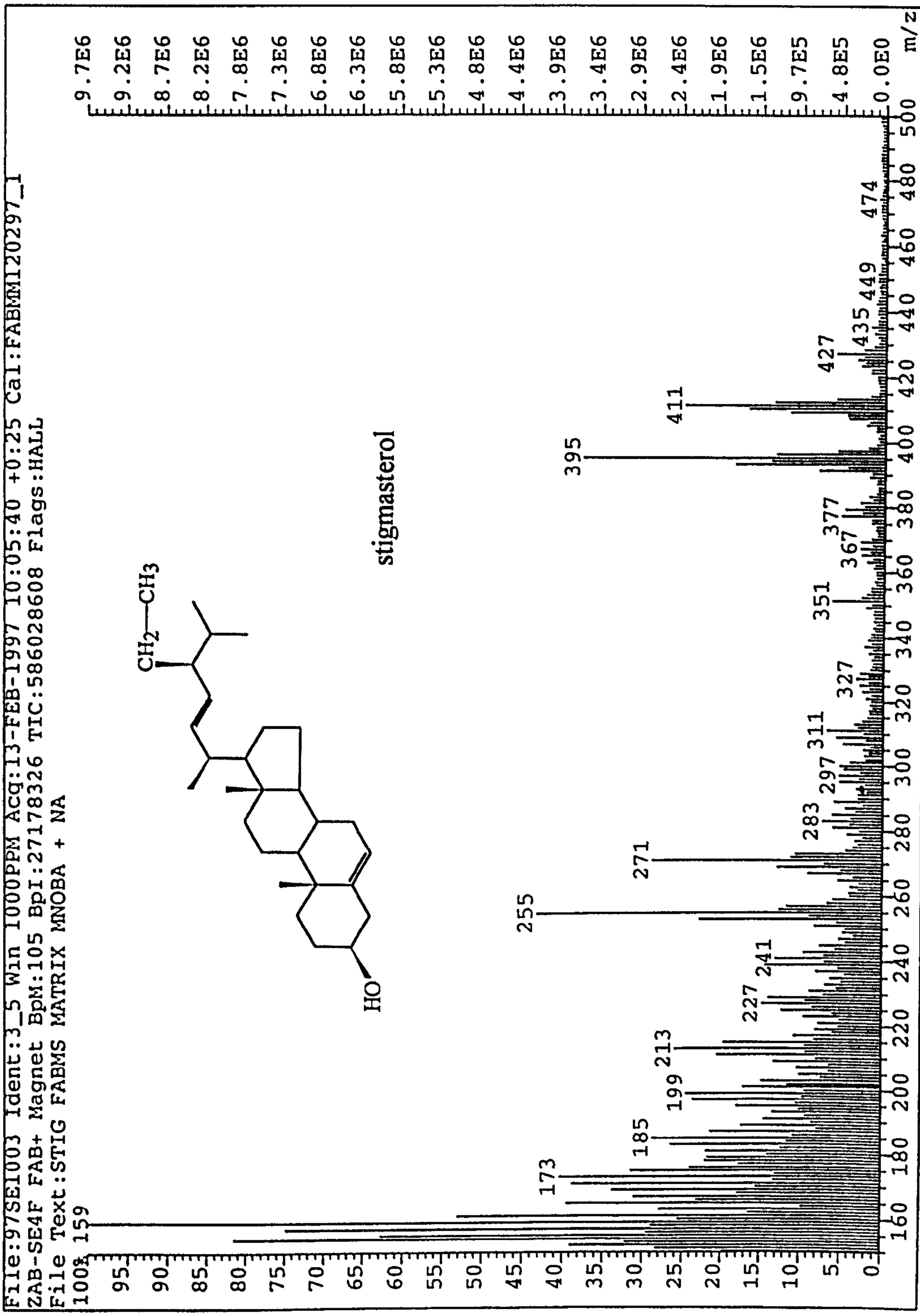
Output m/z range : 33.0000 to 464.7910 Cut Level : 0.00 %

4527977

stigmasterol

d)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449
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199
213
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351
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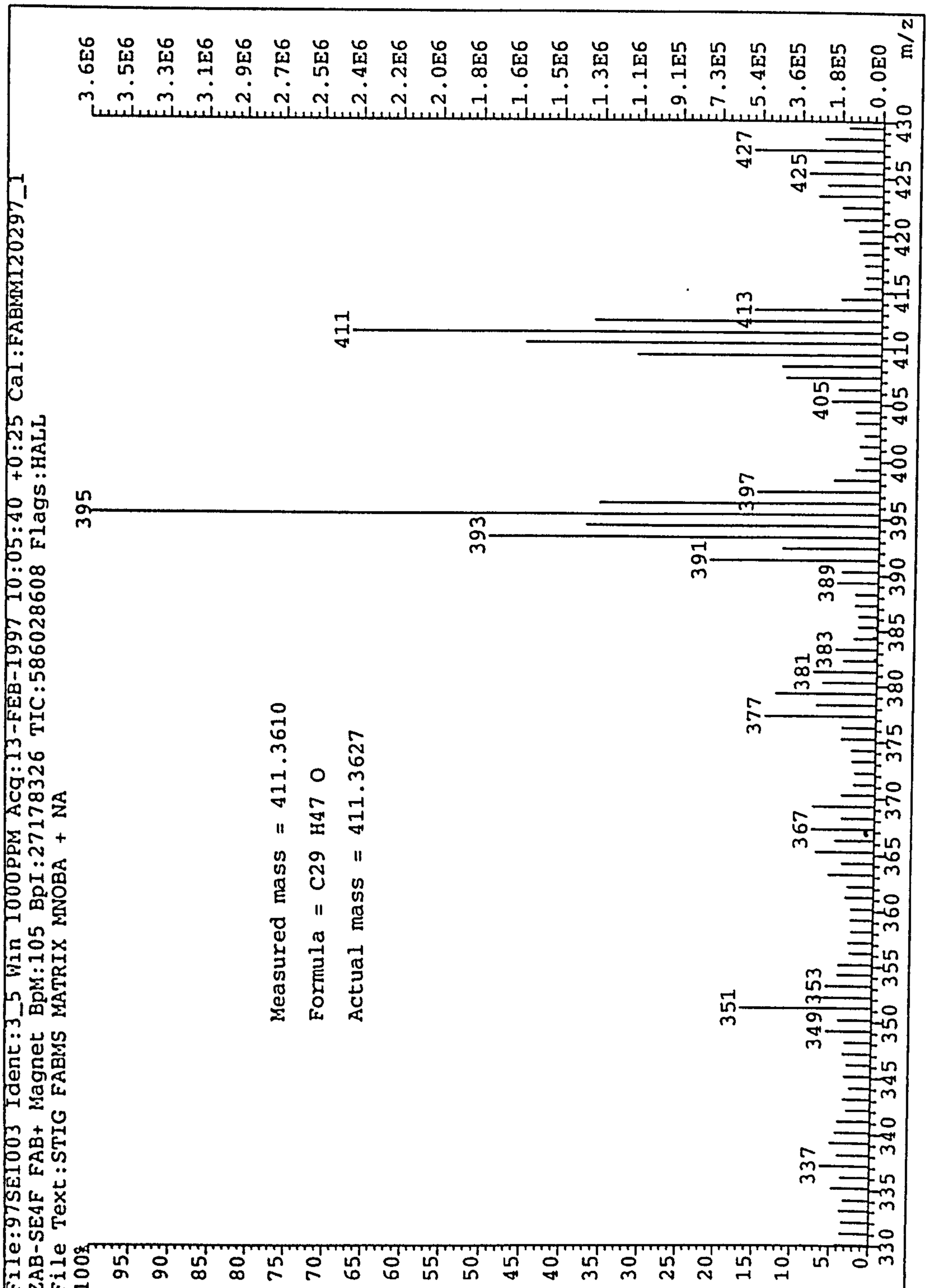
377

d)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ZAB-SE4F FAB+ Magnet BpM:105 BpI:27178326 TIC:586028608 Flags:HALL
File Text:STIG FABMS MATRIX MNOBA + NA
Measured mass = 411.3610
Formula = C29 H47 O
Actual mass = 411.3627
m/z
%

Appendix 9: NMR and MS spectra for compound M2ItC

a) ^{1}H 400 MHz NMR spectrum ($CDCl_3$)

b) ^{13}C 100 MHz NMR spectrum ($CDCl_3$); full spectrum + DEPT

c) COSY (correlated spectroscopy) spectrum ($CDCl_3$)

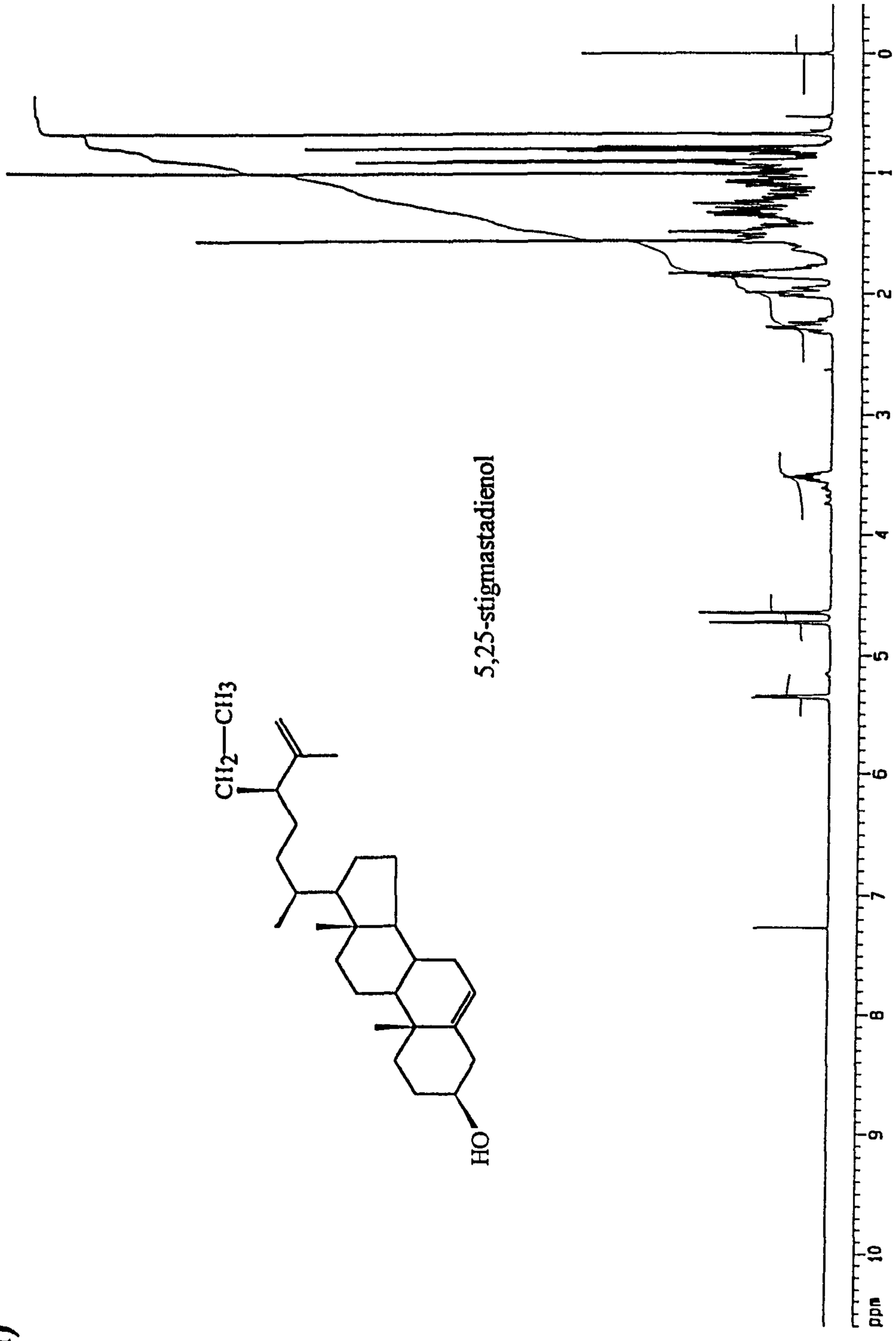
d) NOESY (nuclear overhauser enhancement spectroscopy) spectrum ($CDCl_3$)

e) C-H correlation spectrum ($CDCl_3$)

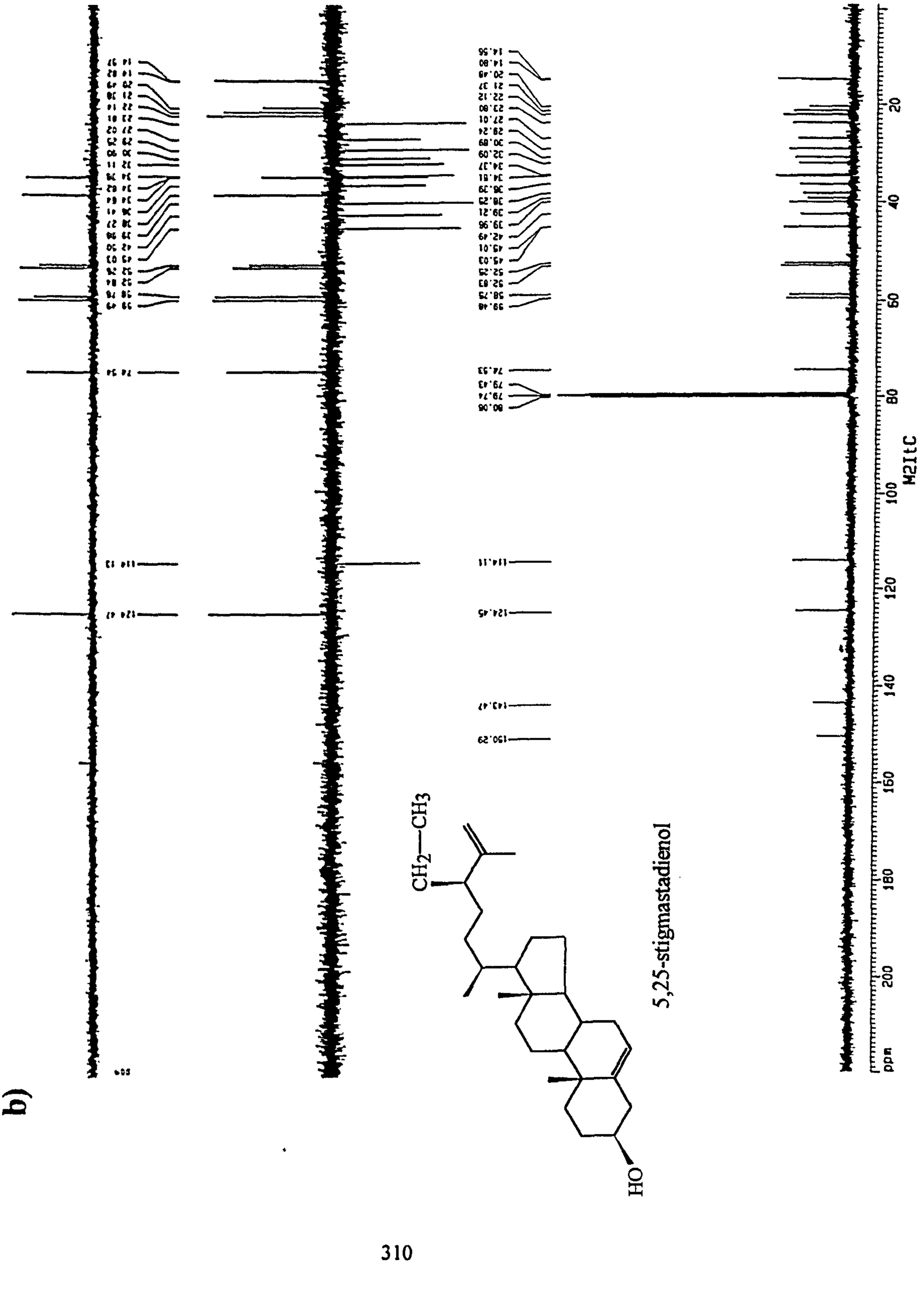
f) Electron impact mass spectrum

g) Fast atom bombardment mass spectrum (matrix: MNOBA + Na)

a)



b)


Current Data Parameters
F2 - Acquisition Parameters
F2 - Processing parameters
1D NMR plot parameters
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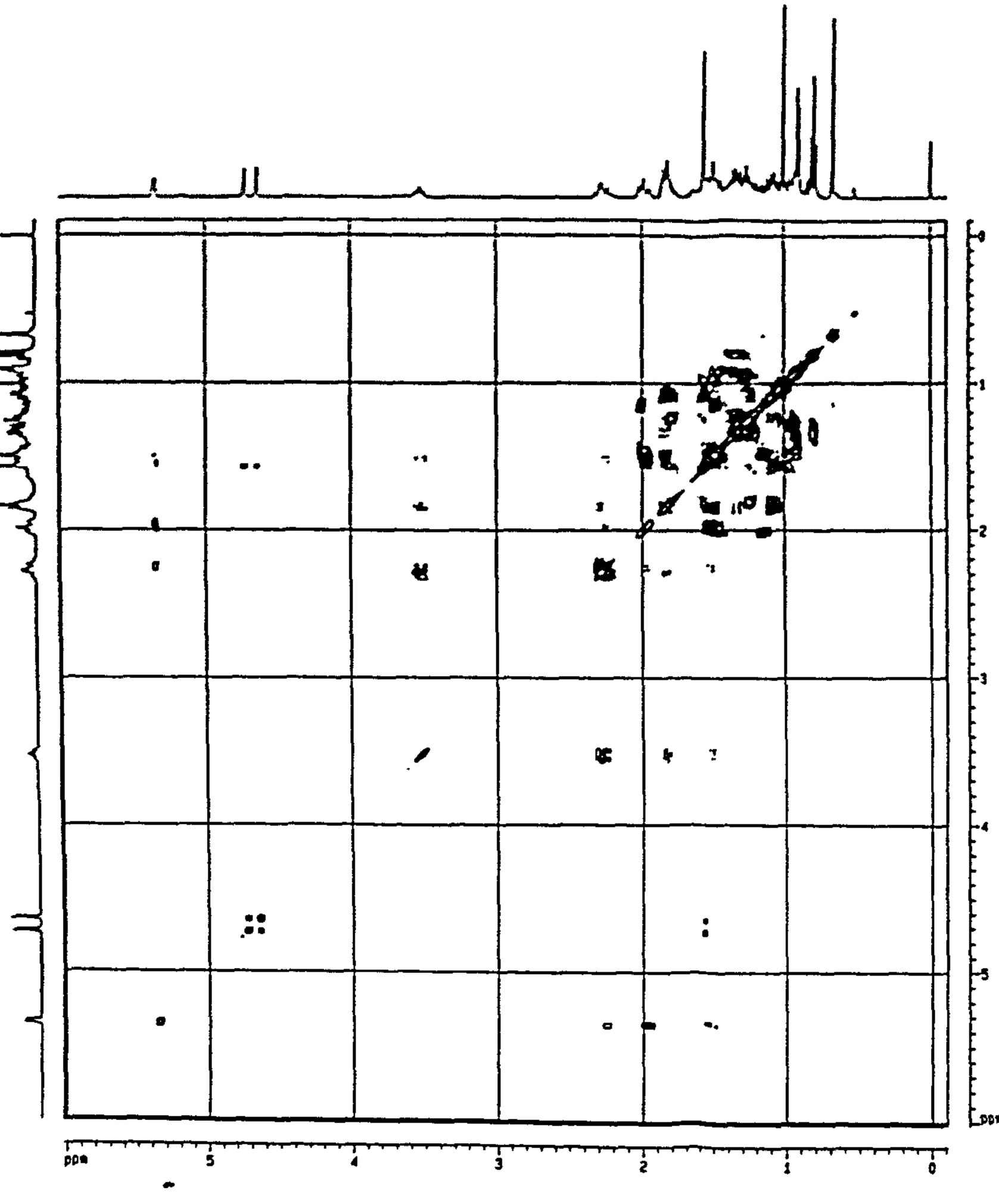
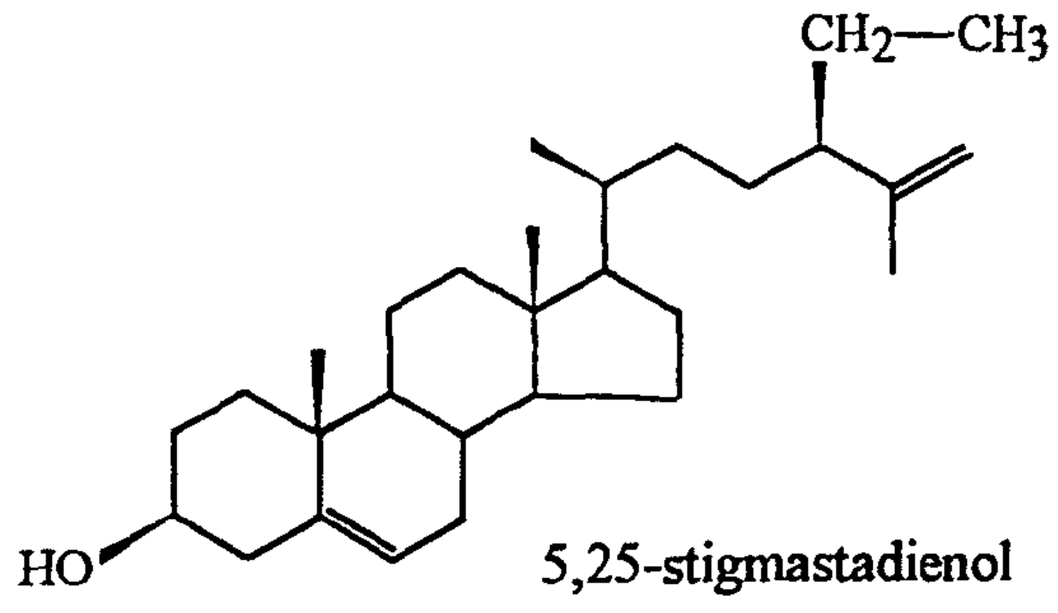
14.82
20.49
21.38
22.14
23.81
27.02
29.25
30.90
32.11
34.38
34.62
34.64
36.41
38.27
39.98
42.30
43.03
52.24
52.84
58.78
59.46
74.34
79.43
80.06
114.13
124.47
124.45
143.47
150.29
14.56
14.80
20.49
21.37
22.12
23.80
27.01
29.24
30.89
32.09
34.37
34.81
36.39
38.23
39.21
39.98
42.49
43.01
45.03
52.25
52.83
58.75
59.48
74.53
79.43
79.74
80.06
114.11
124.45
143.47
150.29
20
40
60
80
100
120
140
160
180
200
ppm
H2O
CH2—CH3
HO
5,25-stigmastadienol

c)





5,25-stigmastadienol

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EXPNO 30
PROCNO 1

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Time 18 14
PULPROG cosygpqf
SOLVENT CDCl3
AQ 0 4179129 sec
DW 204 0 usec
DE 256
NUCLEUS 1H
P1 2 us
D1 0 8306167 sec
P3 0 5 usec
D0 0 0000030 sec
D8 172 9 usec
SFO1 400 1358745 MHz
SWH 2450 98 Hz
TD 2048
NS 8
DS 4
D0 0 0004080 sec

F1 - Acquisition parameters
NDO 1
TD 256
SFO1 400 1358 MHz
FIDRES 9 574142 Hz
SW 6.125 ppm

F2 - Processing parameters
SI 1024
SF 400 1343926 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
SR 4392 62 Hz
HZPT 2.393536 Hz

F1 - Processing parameters
SI 1024
MC2 QF
SF 400 1343926 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0

2D NMR plot parameters
CX2 20 00 cm
CX1 20.00 cm
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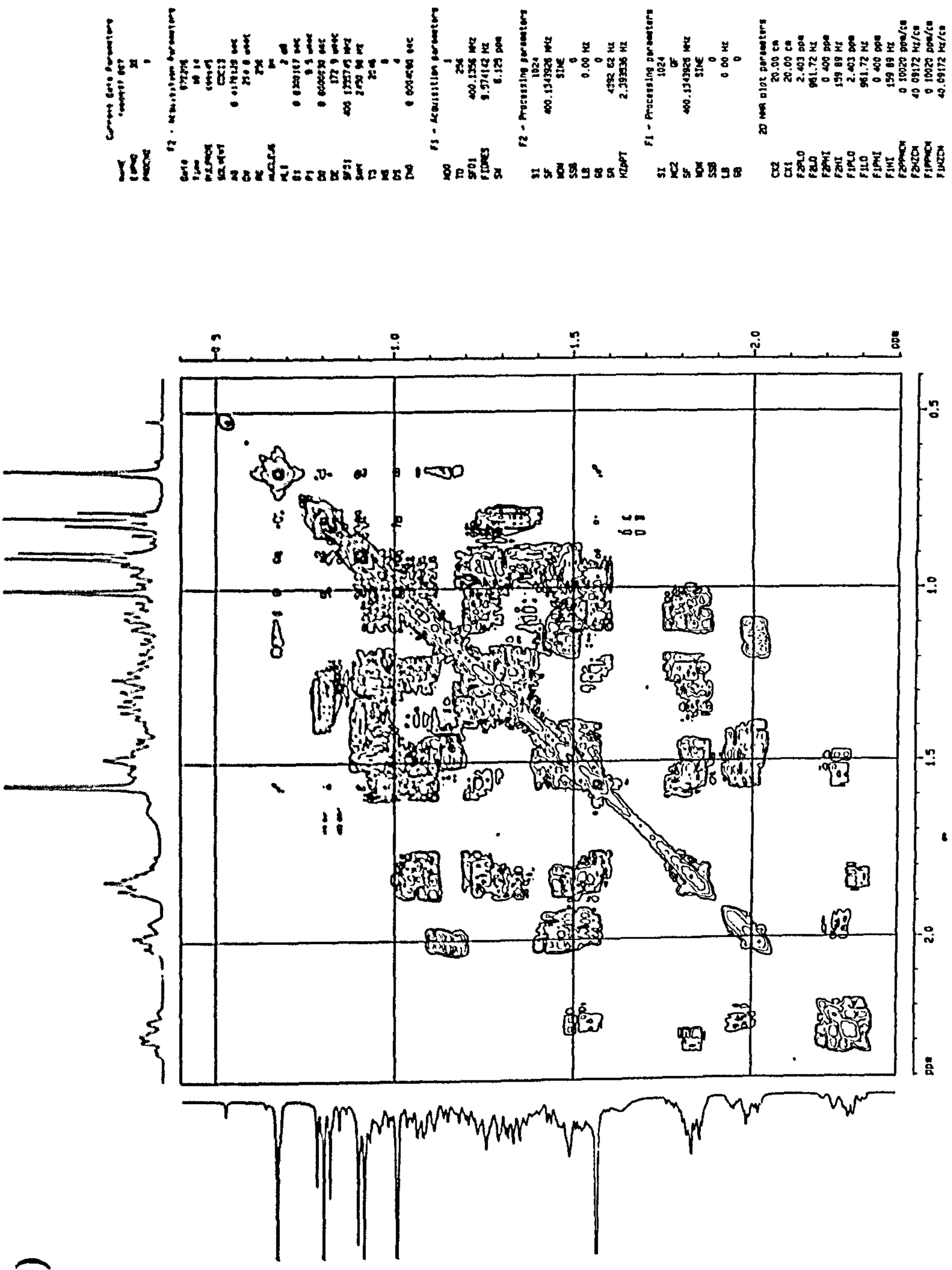
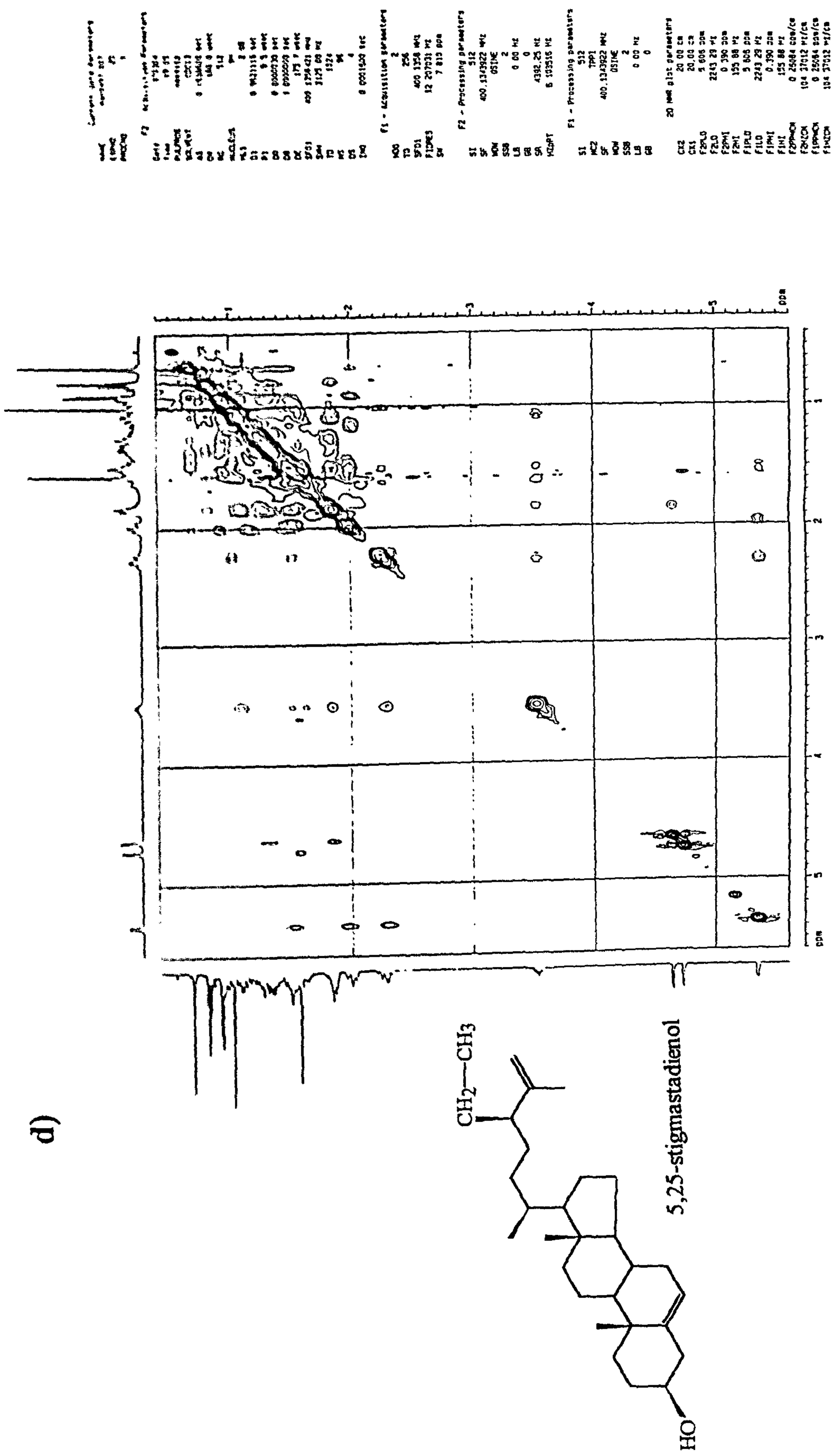
F2LO 2407.41 Hz
F2PHI -0.109 ppm
F2HI -43.56 Hz
F1PLO 6.017 ppm
F1LO 2407.41 Hz
F1PHI -0.109 ppm
F1HI -43 56 Hz
F2PPMCM 0 30627 ppm/cm
F2HZCM 122.54868 Hz/cm
F1PPMCM 0 30627 ppm/cm
F1HZCM 122 54868 Hz/cm


c)
0.5
1.0
1.5
2.0
ppm

d)

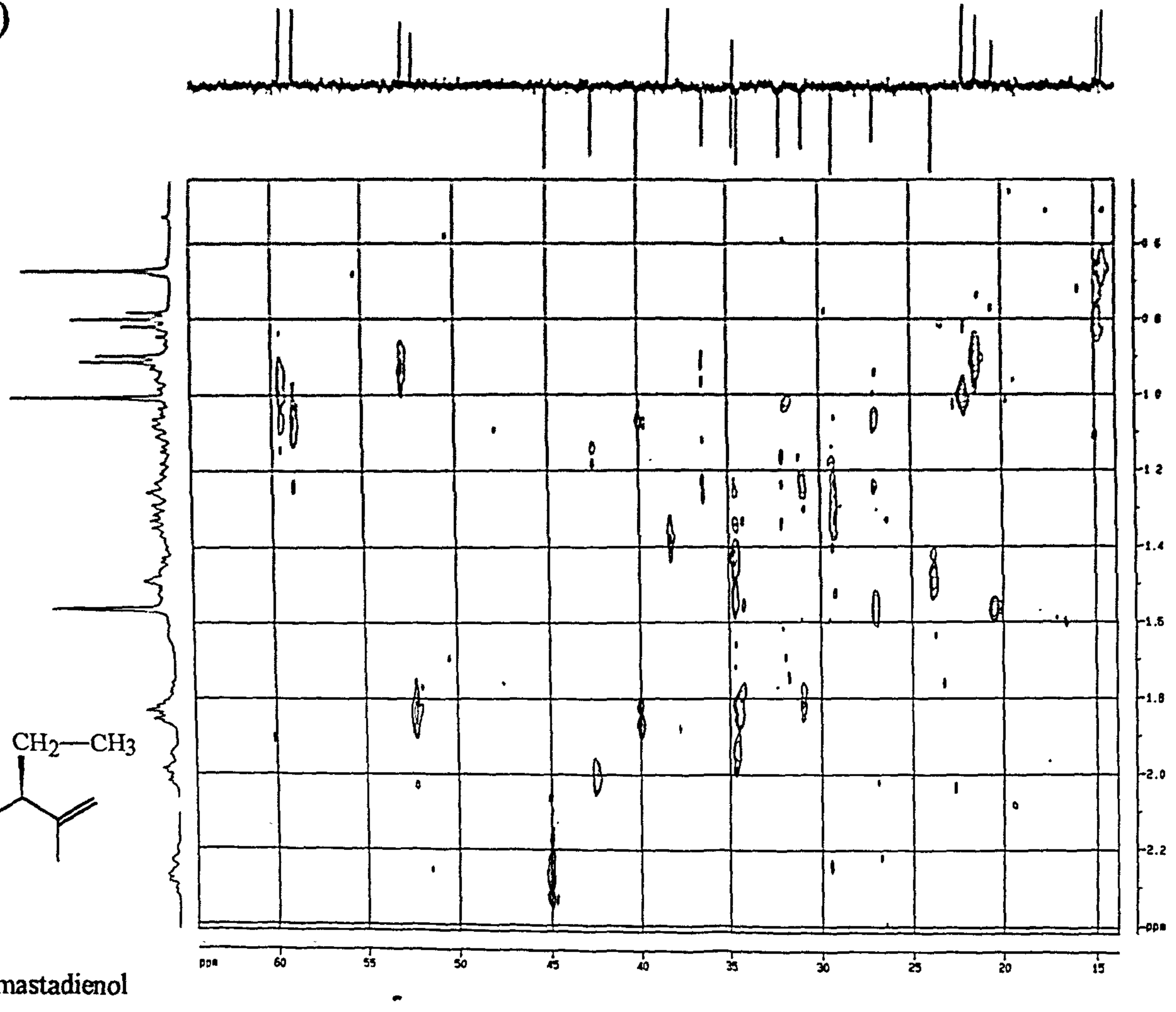


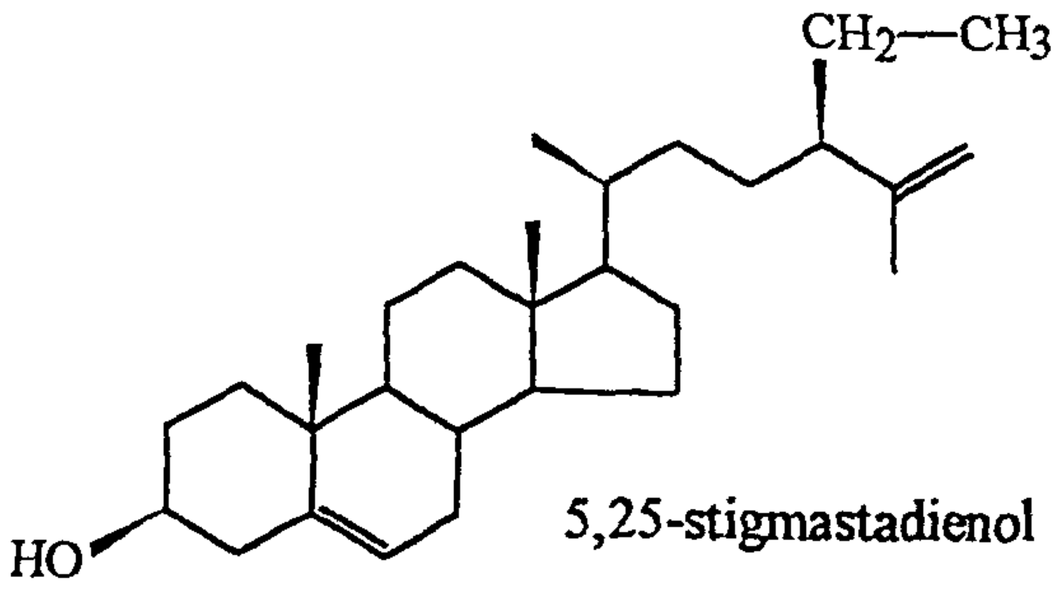
$CH_2—CH_3$

5,25-stigmastadienol

HO

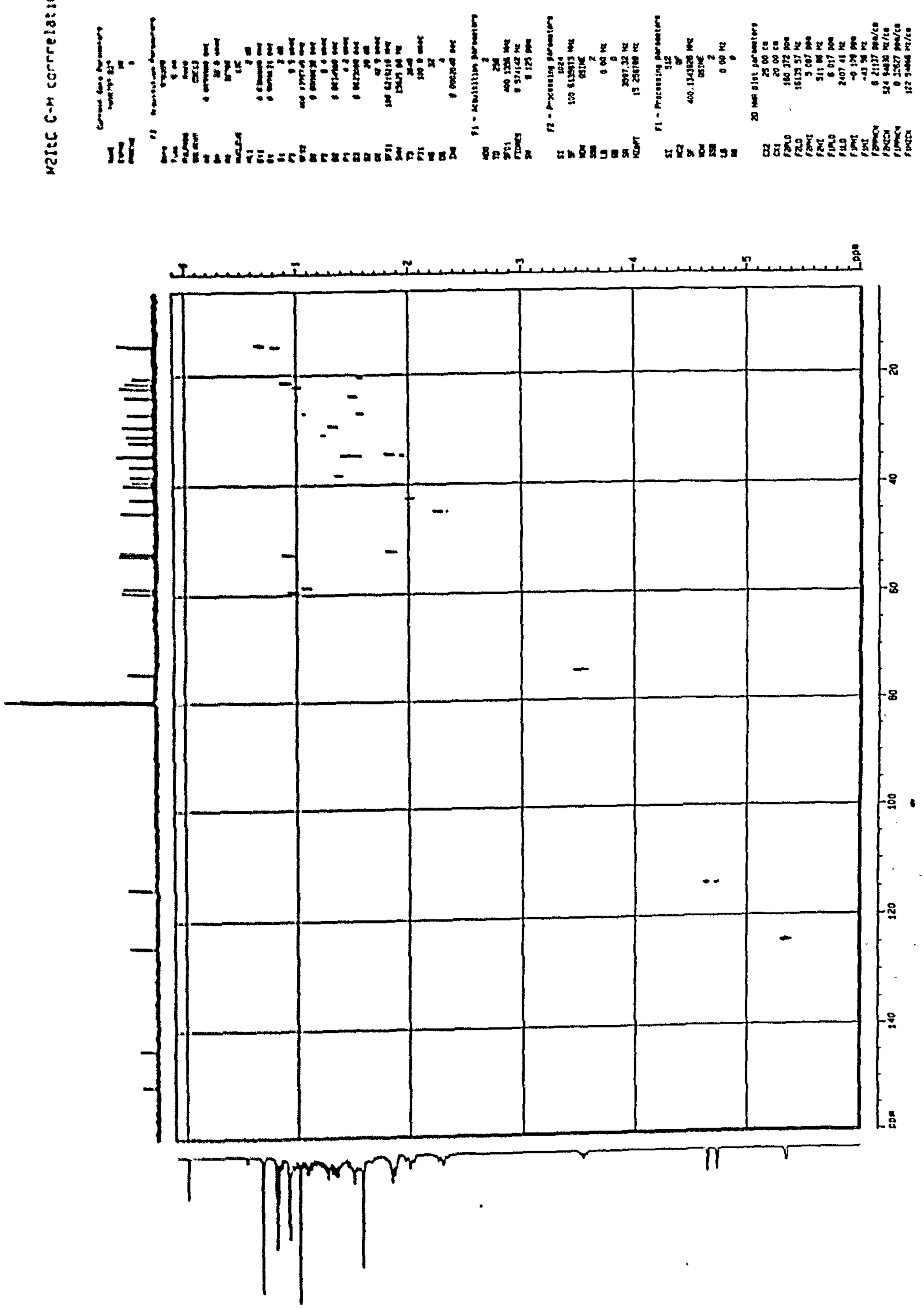
e)



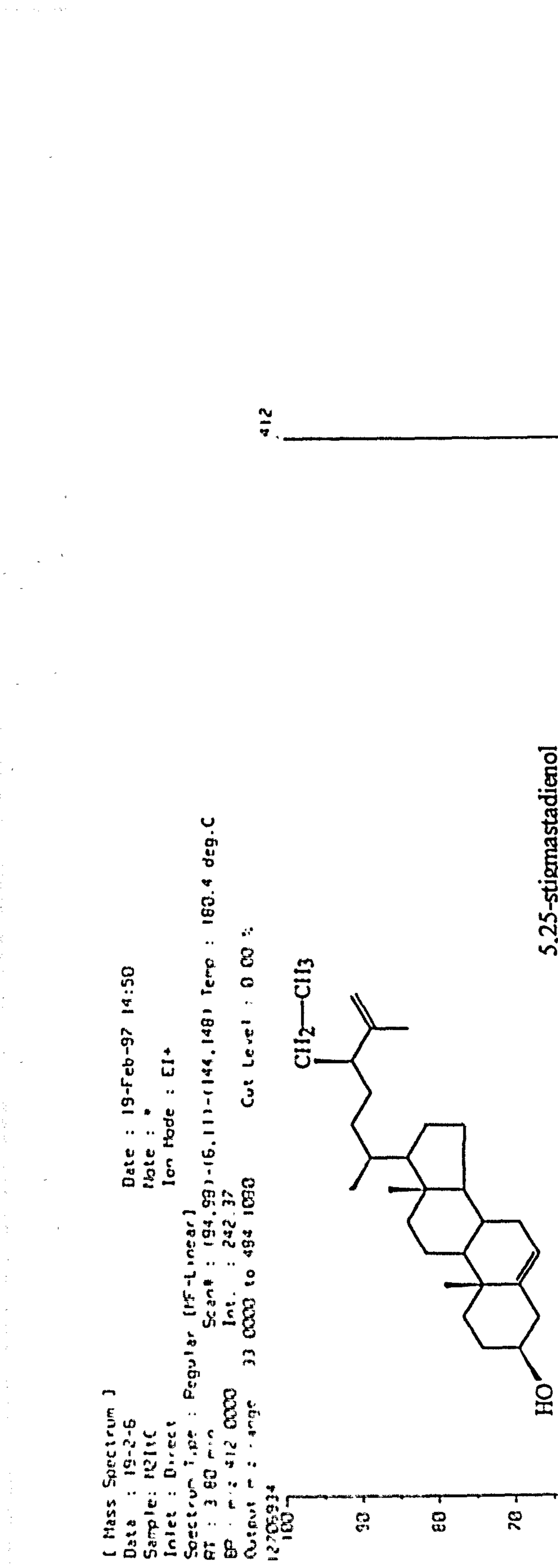


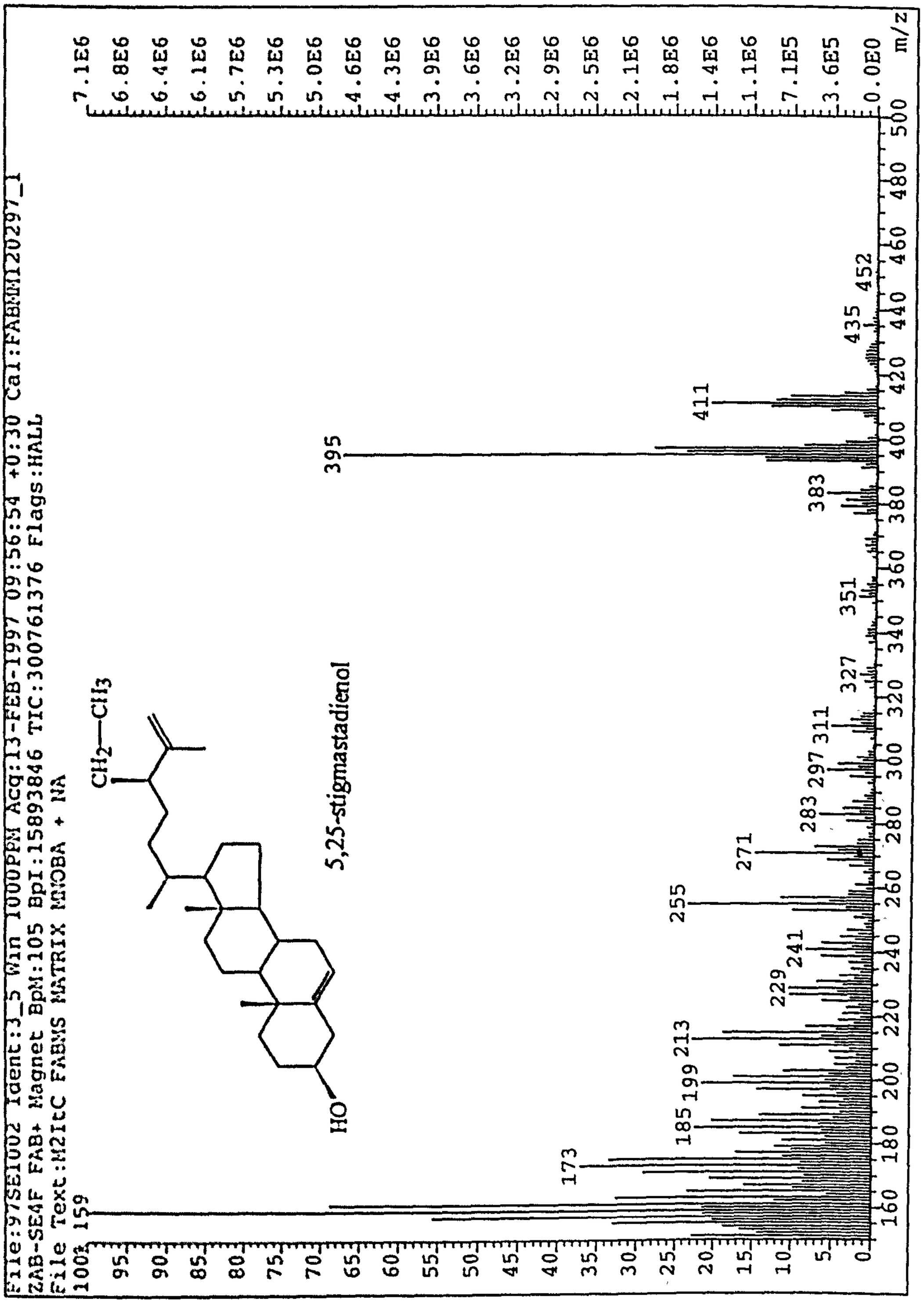
5,25-stigmastadienol

H2ItC C-H correlation


ppm
e)

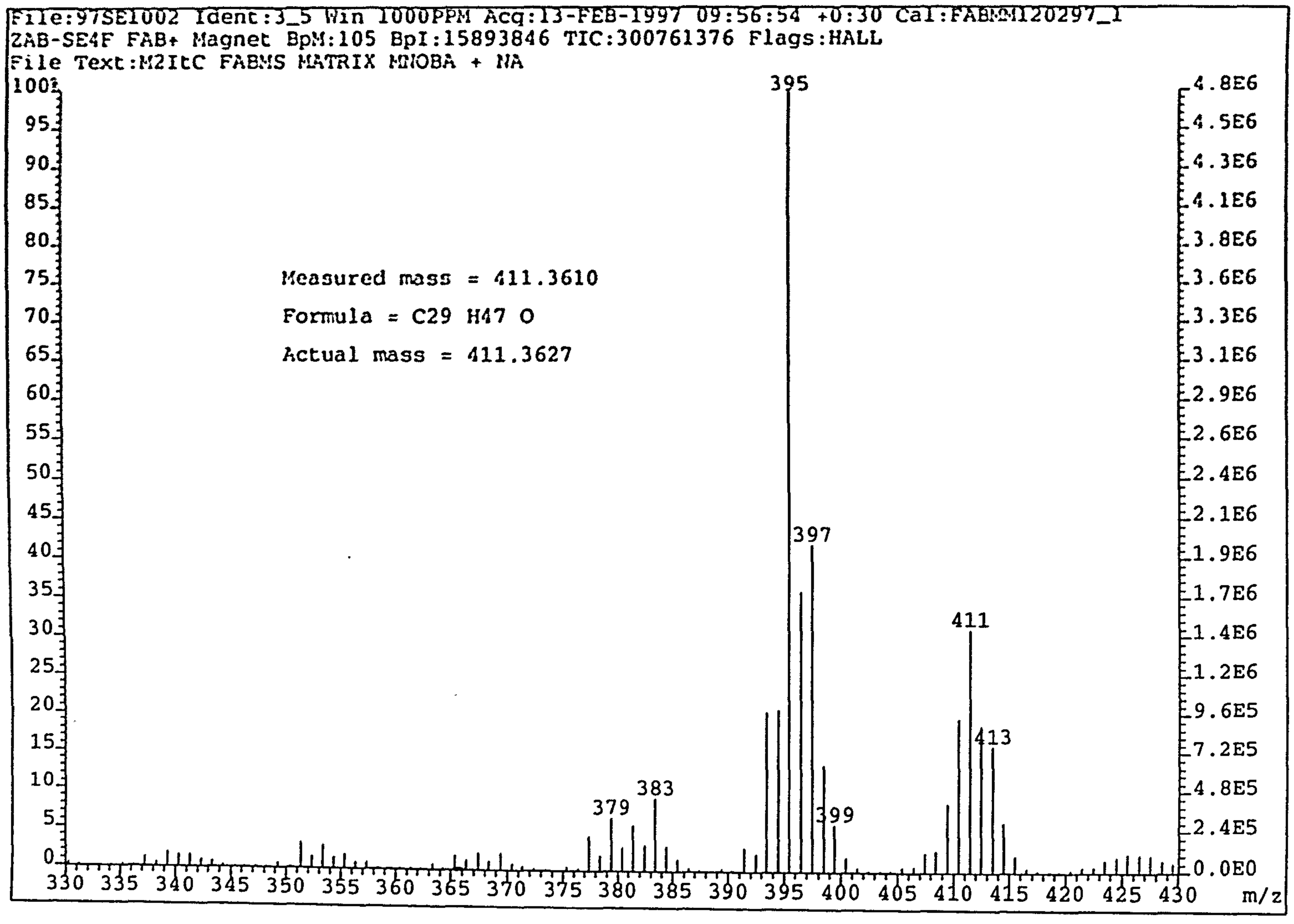
f)


(Mass Spectrum)
Date : 19-Feb-97 14:50
Sample: H21C-6
Note : *
Inlet : Direct
Ion Mode : EI+
Spectrum Type : Regular (MF-Linear)
RT : 3.80 min Scan# : 194.69)-(6.111-(144.148) Temp : 180.4 deg.C
BP : m/z 412.0000 Int. : 242.37
Output : 33.0000 to 484.1000 Cut Level : 0.00 %
412.0000
100
90
80
70
60
50
40
30
20
10
0
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
m/z
55
81
107
123
145
159
199
213
231
255
271
299
328
353
379
397
412
5,25-stigmastadienol
CH_2—CH_3
HO


File:975E1002 Ident:3_5 Win 1000PPM Acq:13-FEB-1997 09:56:54 +0:30 Cal:FABEI20297
ZAB-SE4F FAB+ Magnet BpM:105 BpI:15893846 TIC:30076761376 Flags:HALL
File Text:M2ItC FABMS MATRIX MNOBA + NA
5,25-stigmastadienol
HO
CH2—CH3
m/z
395
411
383
351
435
452
255
271
283
297
311
327
173
185
199
213
229
241

g)

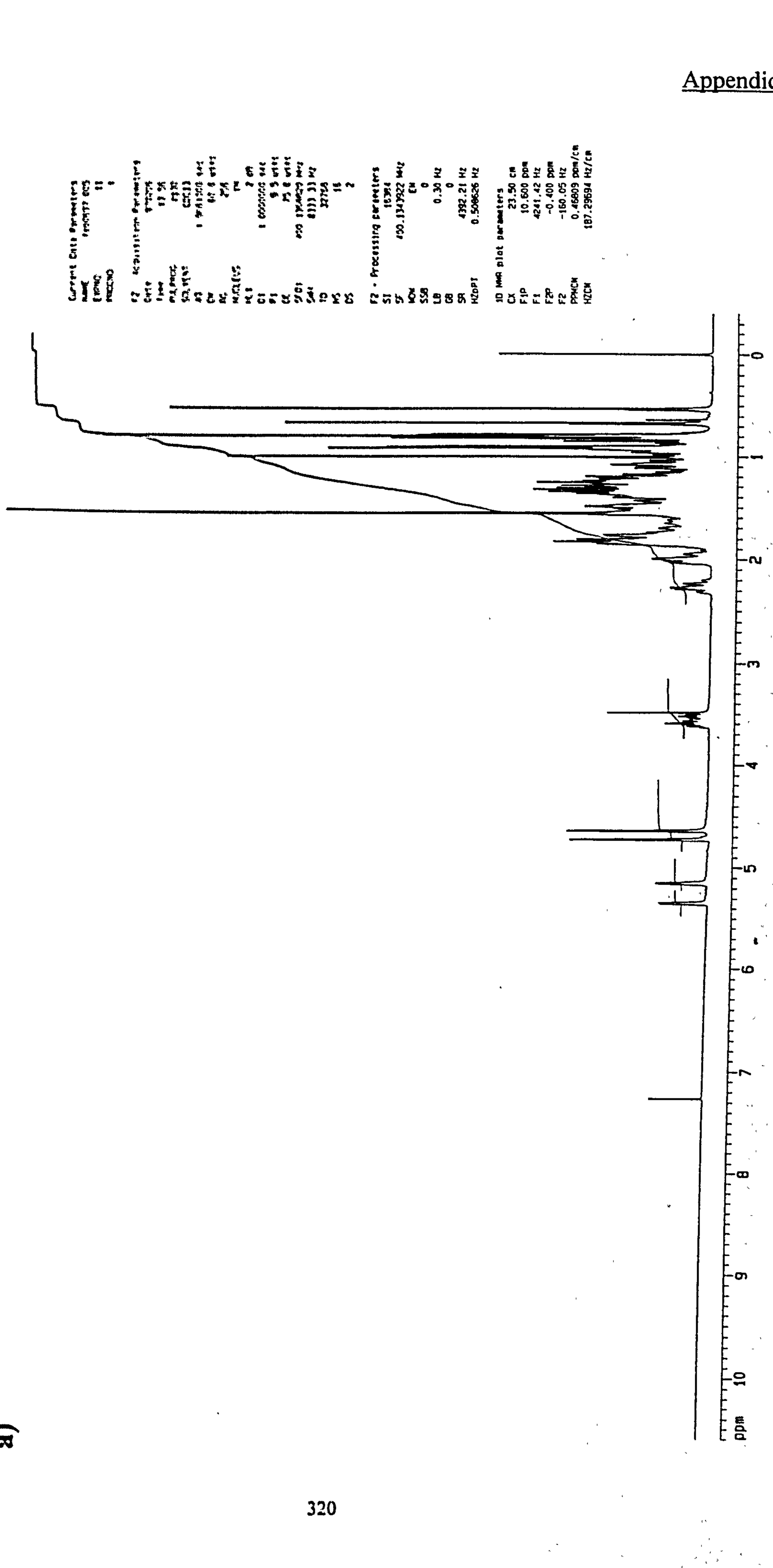
g)


File:97SE1002 Ident:3_5 Win 1000PPM Acq:13-FEB-1997 09:56:54 +0:30 Cal:FABMM120297_1
ZAB-SE4F FAB+ Magnet BpM:105 BpI:15893846 TIC:300761376 Flags:HALL
File Text:M2ItC FABMS MATRIX MNOBA + NA
Measured mass = 411.3610
Formula = C29 H47 O
Actual mass = 411.3627
395
397
411
413
379
383
399
m/z

Appendix 10: NMR spectra for compound M2ItB

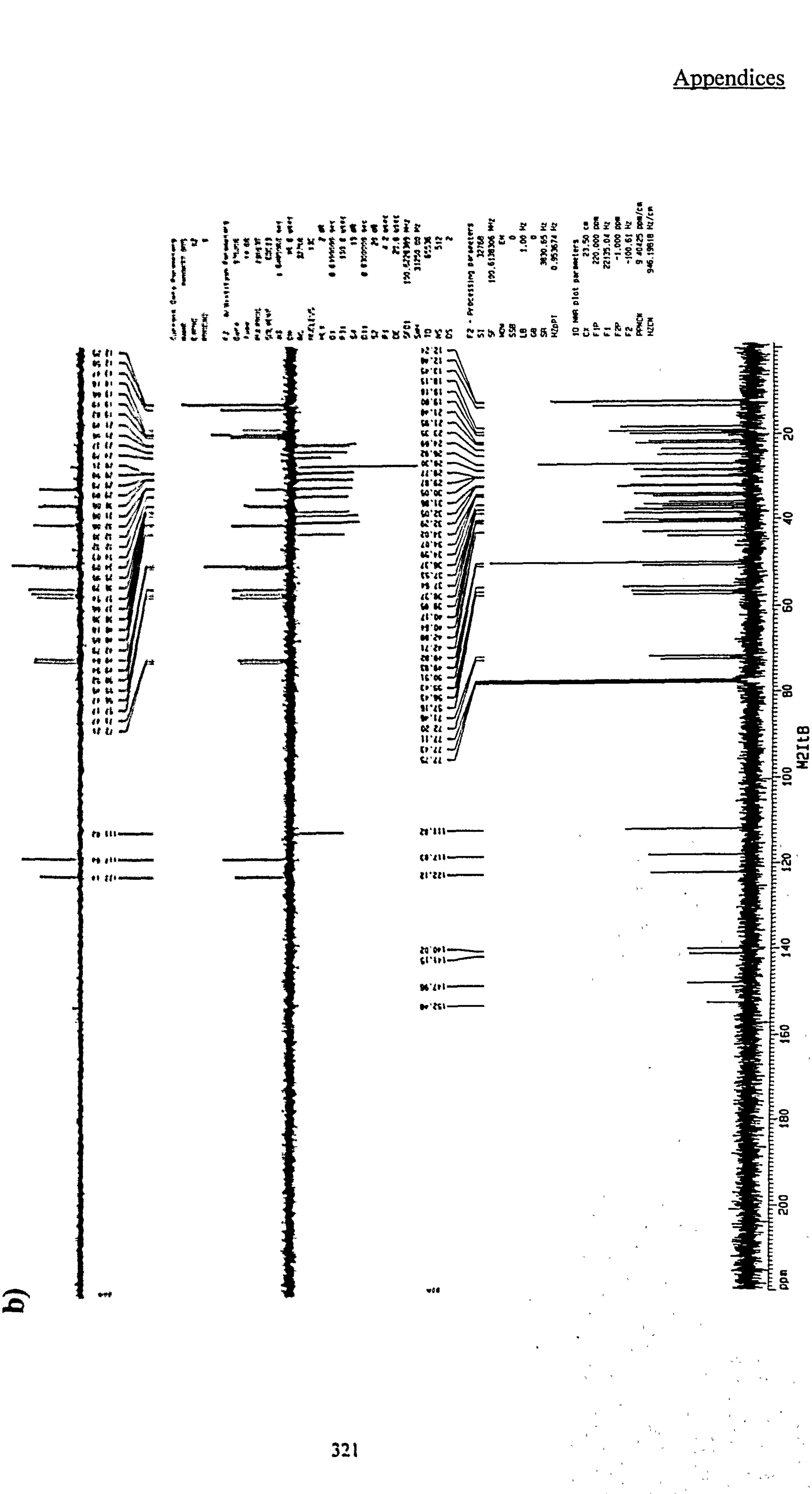
a) ^{1}H 400 MHz NMR spectrum ($CDCl_3$)

b) ^{13}C 100 MHz NMR spectrum ($CDCl_3$); full spectrum + DEPT


Current Data Parameters
F2 - Acquisition Parameters
F2 - Processing parameters
1D NMR plot parameters
M2It8
ppm
0
1
2
3
4
5
6
7
8
9
10

a)

Appendices



Appendix 11: GC-MS analysis data

a) Electron impact mass spectrum for β-sitosterol

b) Electron impact mass spectrum for 5,25-stigmastadienol

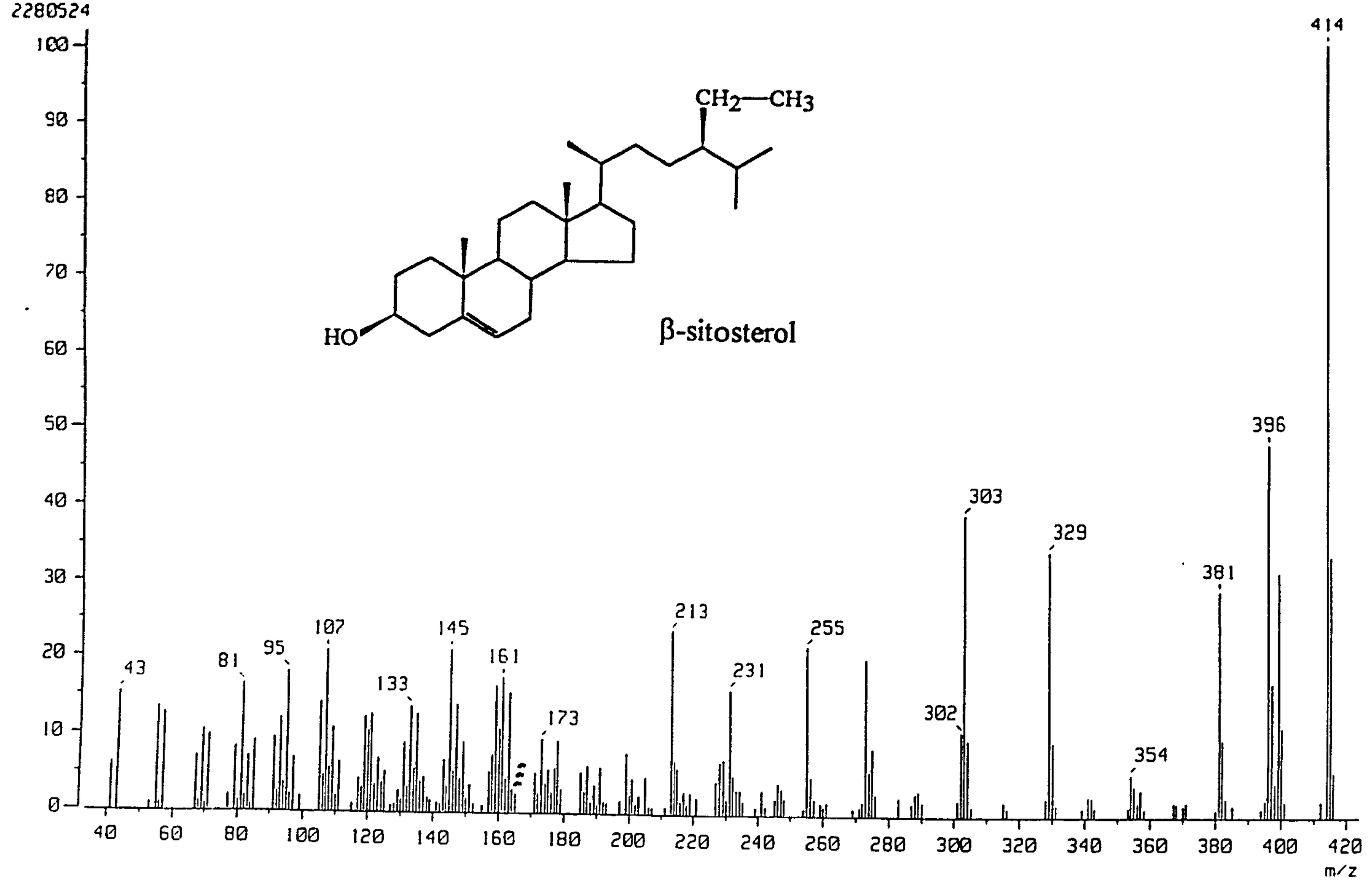
c) Electron impact mass spectrum for stigmasterol

d) Elution rate of the three reference compounds

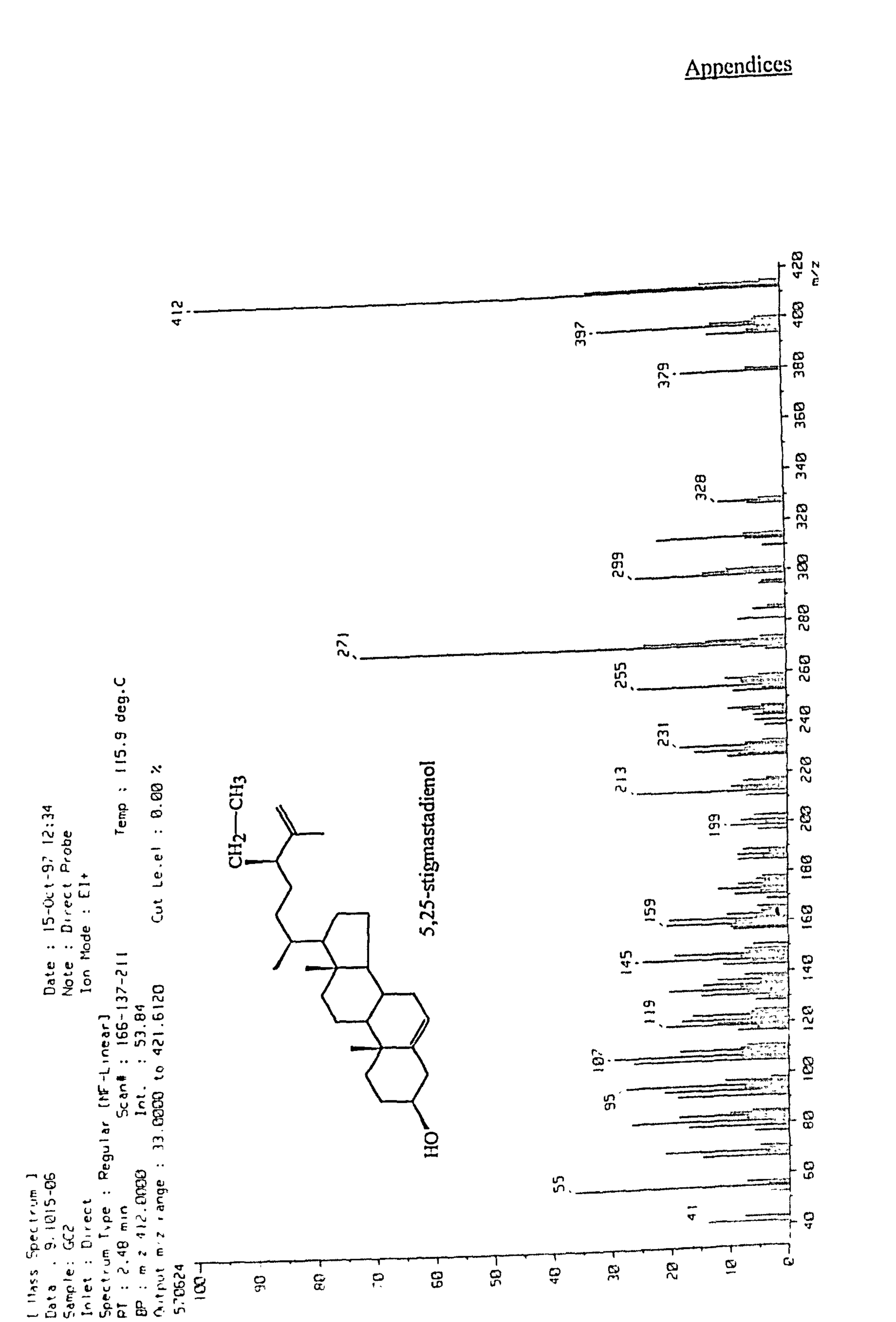
a)

[Mass Spectrum]
Data : 971015-05 Date : 15-Oct-97 12:16
Sample: GC1 Note : Direct Probe
Inlet : Direct Ion Mode : EI+
Spectrum Type : Regular (NF-Linear)
RT : 2.73 min Scan# : 183 Temp : 111.7 deg.C
BP : m/z 414.0000 Int. : 212.91
Output m/z range : 33.0000 to 423.8840 Cut Level : 0.00 %

2280524

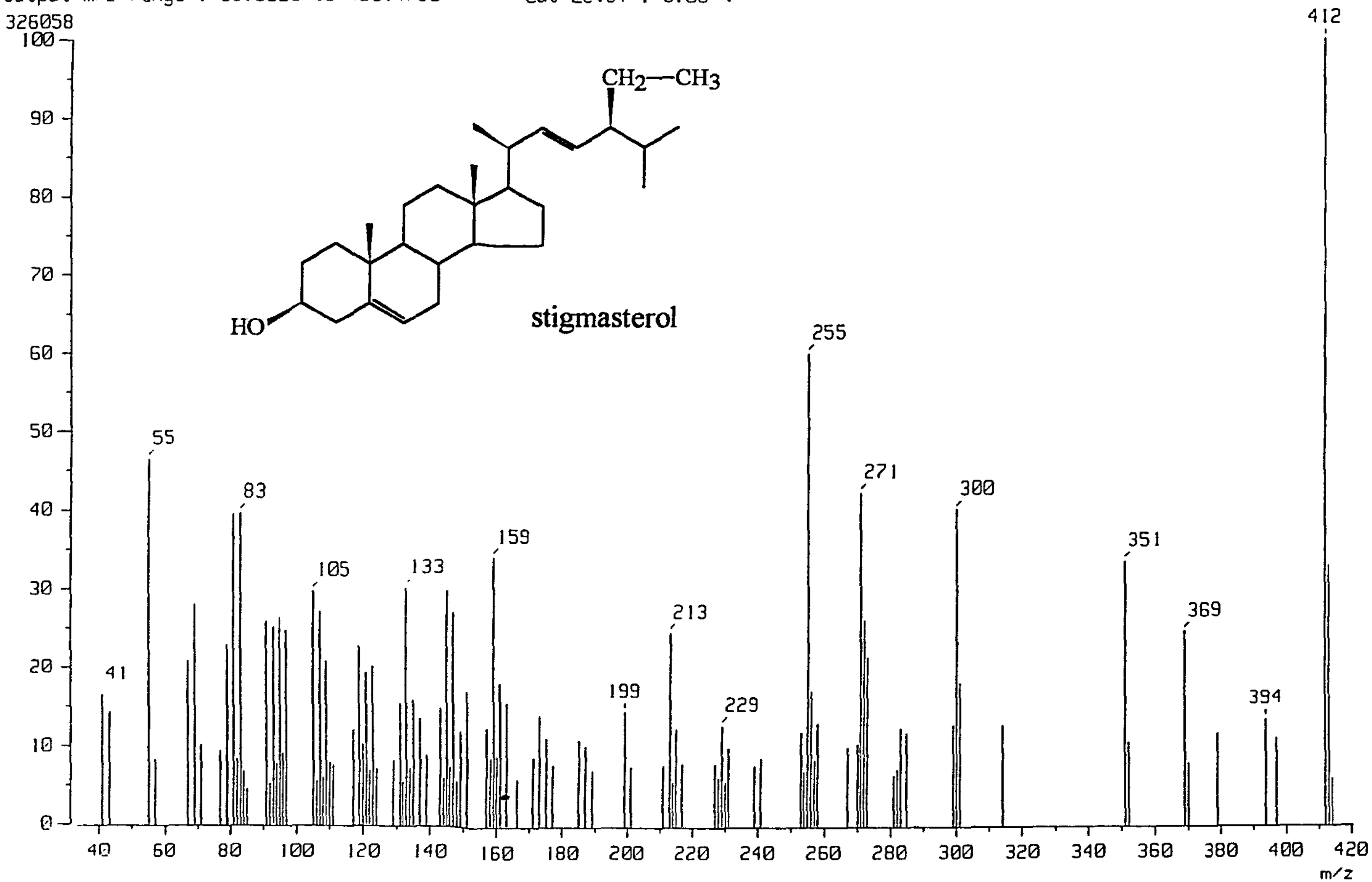


b)

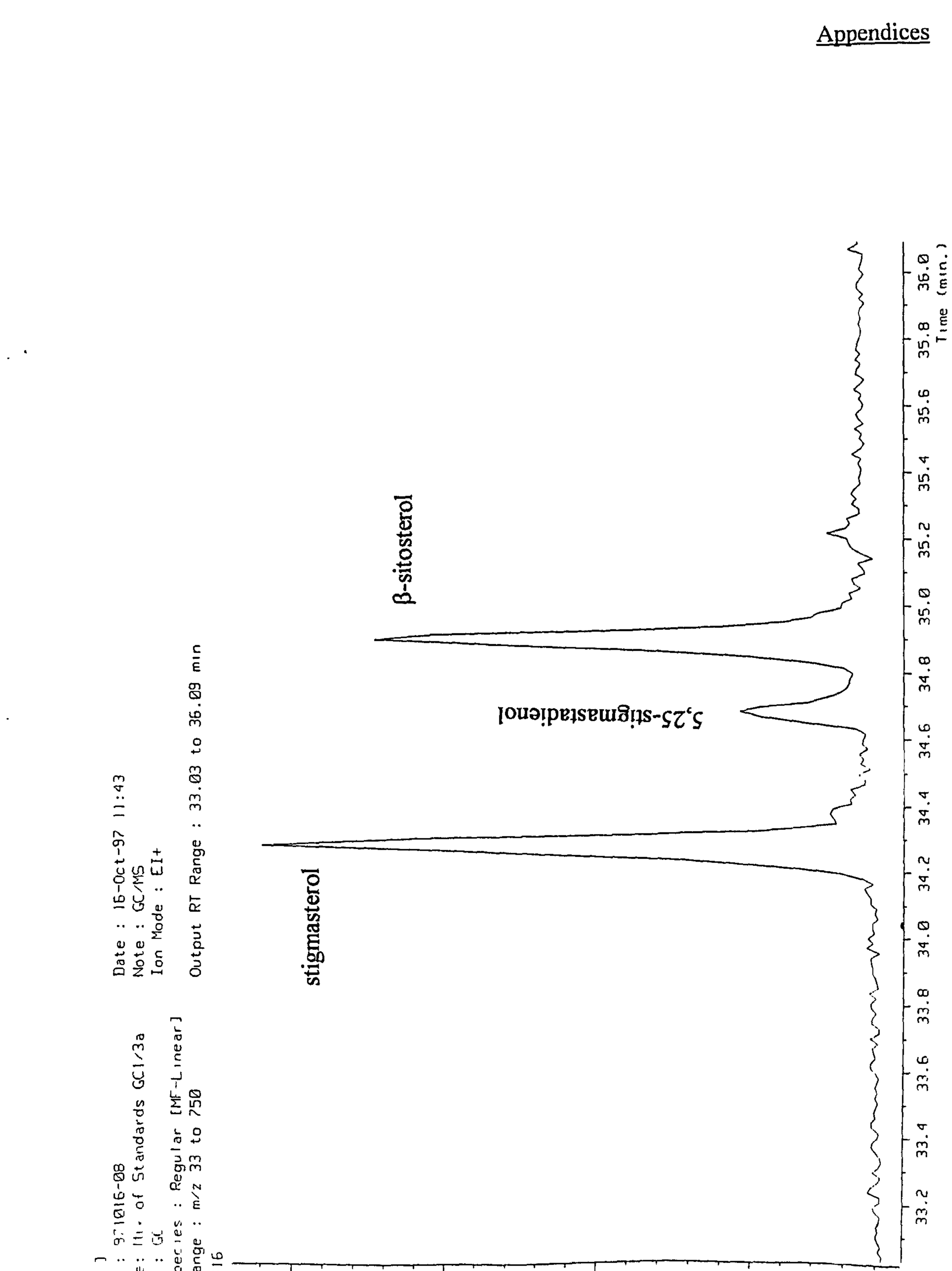

Date : 15-Oct-97 12:34
Note : Direct Probe
Ion Mode : EI+
Temp : 115.9 deg.C
Cut Level : 0.00 %
Cut
Spectrum Type : Regular (NF-Linear)
Scan# : 166-137-211
Int. : 53.84
BP : m/z 412.0000
m/z range : 33.0000 to 421.6120
5,25-stigmastadienol
HO
CH2—CH3
m/z
412
397
379
328
299
271
255
231
213
199
159
145
119
107
95
69
55
41

c)

[Mass Spectrum]
Data : 971015-07 Date : 15-Oct-97 12:54
Sample: GC3A Note : Direct Probe
Inlet : Direct Ion Mode : EI+
Spectrum Type : Regular [MF-Linear]
RT : 2.22 min Scan# : 149-91-222 Temp : 104.0 deg.C
BP : m/z 412.0000 Int. : 31.10
Output m/z range : 33.0000 to 420.4750 Cut Level : 0.00 %



d)


[TIC]
Data : 971016-08
Sample: 11- of Standards GC1/3a
Inlet : GC
Ion Species : Regular [MF-Linear]
TIC Range : m/z 33 to 750
12973416
Date : 16-Oct-97 11:43
Note : GC/MS
Ion Mode : EI+
Output RT Range : 33.03 to 36.09 min
stigmasterol
5,25-stigmastadienol
β-sitosterol
Time (min.)

Appendix 12: NMR and MS spectra for compound M2Ciii

a) ^{1}H 400 MHz NMR spectrum ($CDCl_3$)

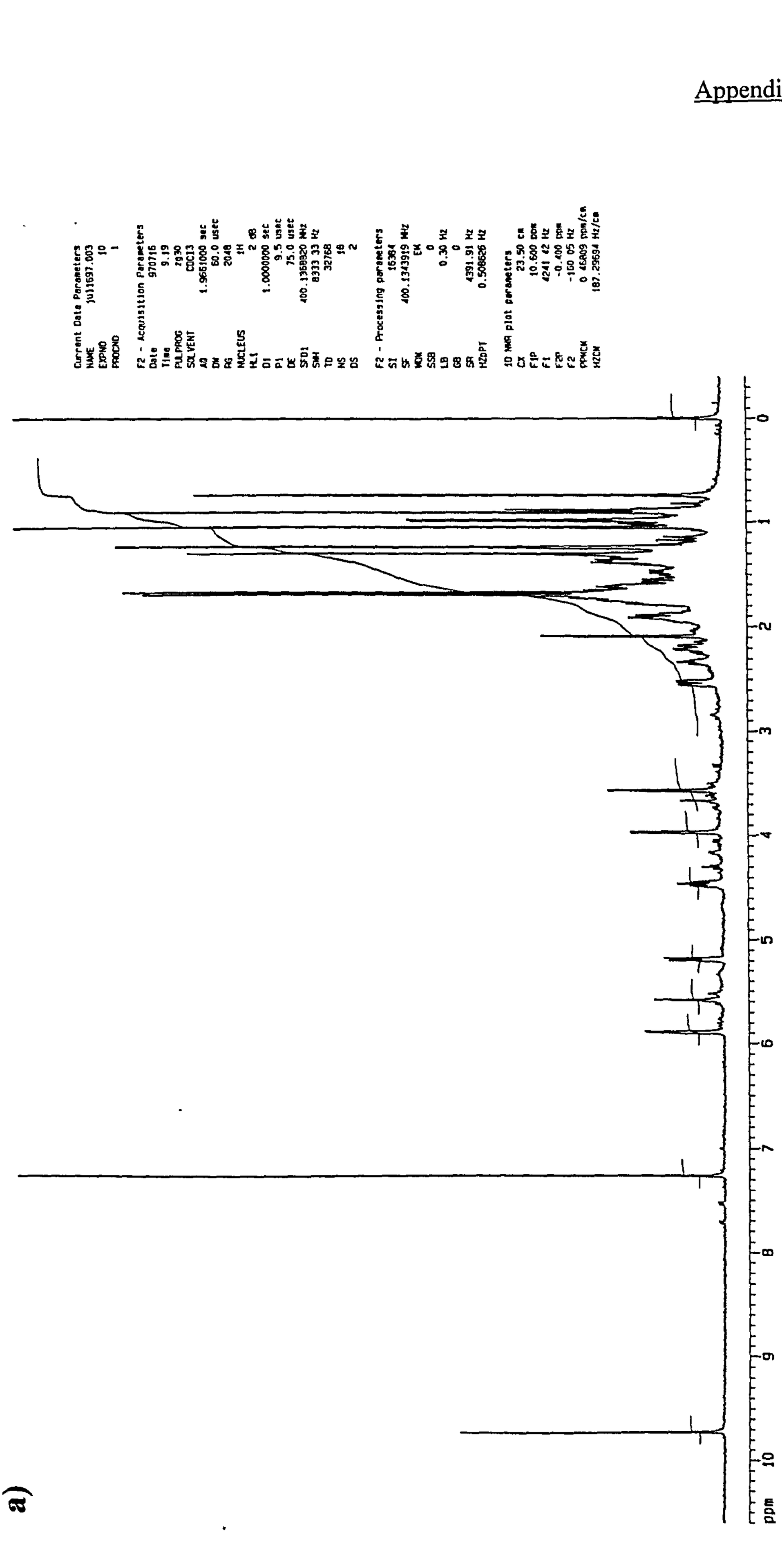
b) ^{13}C 100 MHz NMR spectrum ($CDCl_3$); full spectrum + DEPT

c) COSY (correlated spectroscopy) spectrum ($CDCl_3$)

d) NOESY (nuclear overhauser enhancement spectroscopy) spectrum ($CDCl_3$)

e) Electron impact mass spectrum

f) Fast atom bombardment mass spectrum (matrix: MNOBA + Na)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PROCNO 1
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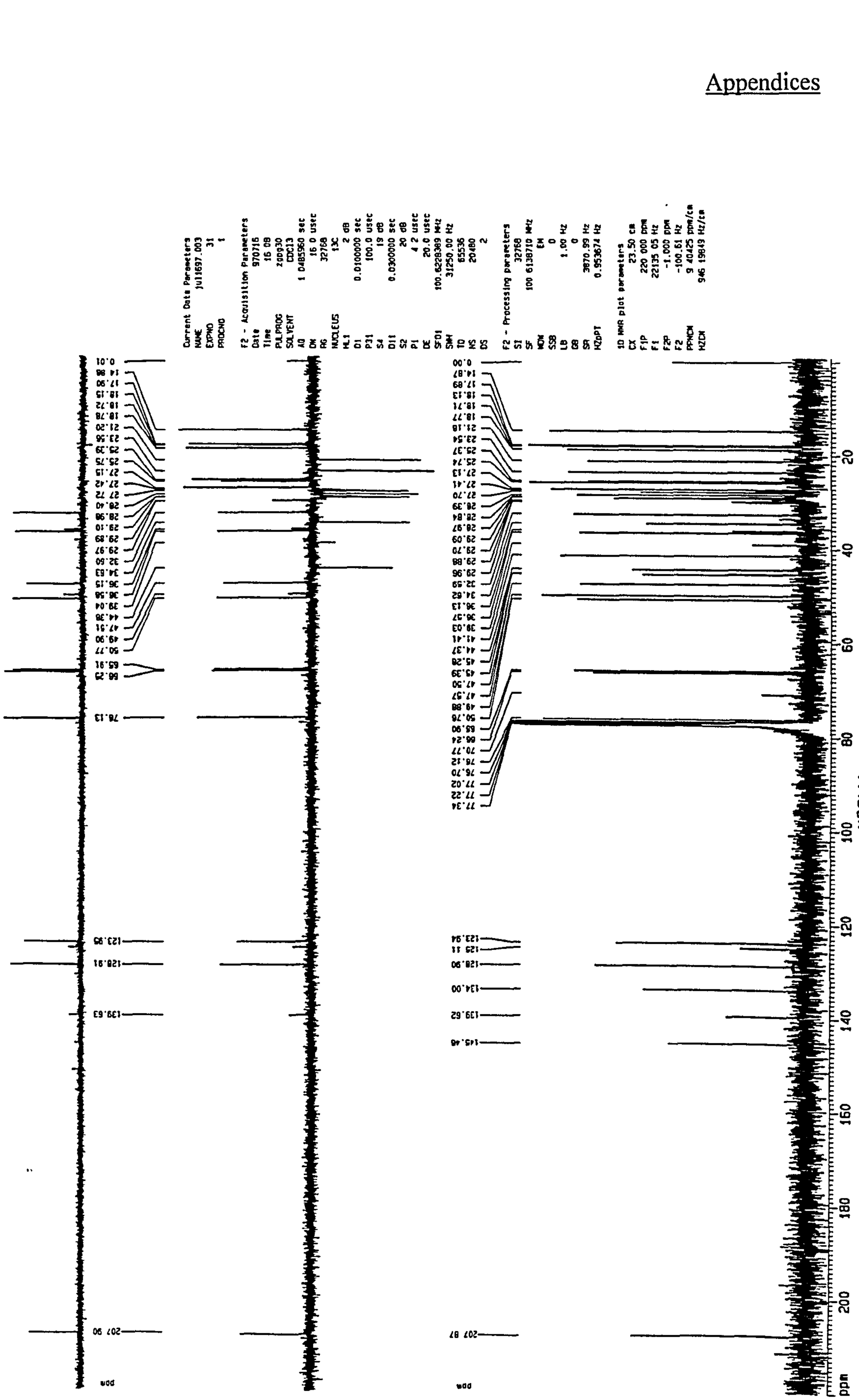
SOLVENT CDCl3
AQ 1.9661000 sec
DW 60.0 usec
RG 2048
NUCLEUS 1H
HL1 2 dB
D1 1.0000000 sec
P1 9.5 usec
DE 75.0 usec
SFO1 400.1368820 MHz
SWH 8333.33 Hz
TD 32768
NS 16
DS 2
F2 - Processing parameters
SI 16384
SF 400.1343919 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
SR 4391.91 Hz
HZDPT 0.508626 Hz
1D NMR plot parameters
CX 23.50 cm
F1P 10.600 ppm
F1 4241.42 Hz
F2P -0.400 ppm
F2 -160.05 Hz
PPMCM 0.45809 ppm/cm
HZCM 187.29694 Hz/cm
M2Ciii
0
1
2
3
4
5
6
7
8
9
10
ppm

a)


Current Data Parameters
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PROCNO 1
F2 - Acquisition Parameters
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Time 16 08
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SOLVENT CDCl3
AQ 1 0485960 sec
DW 16 0 usec
RG 32768
NUCLEUS 13C
HL1 2 dB
D1 0.0100000 sec
P31 100.0 usec
S4 19 dB
D11 0.0300000 sec
S2 20 dB
P1 4 2 usec
DE 20.0 usec
SFO1 100.6228369 MHz
SWH 31250.00 Hz
TD 65536
NS 20480
DS 2
F2 - Processing parameters
SI 32768
SF 100 6138710 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
SR 3870.99 Hz
HZDPT 0.953674 Hz
1D NMR plot parameters
CX 23.50 cm
F1P 220 000 ppm
F1 22135 05 Hz
F2P -1.000 ppm
F2 -100.61 Hz
PPMCM 9 40425 ppm/cm
HZCM 946 19849 Hz/cm
0.01
14.86
17.90
18.15
18.72
18.78
21.20
23.56
25.39
25.75
27.15
27.42
27.72
28.40
28.96
29.10
29.89
29.97
32.60
34.63
36.15
36.56
39.04
44.38
47.91
49.90
50.77
65.91
66.23
78.13
123.95
128.91
139.63
0.00
14.87
17.89
18.13
18.71
18.77
21.18
23.54
25.37
25.74
27.13
27.41
27.70
28.39
28.84
28.97
29.09
29.70
29.88
29.96
32.59
34.62
36.13
36.57
38.03
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44.37
45.28
45.39
47.50
47.57
49.88
50.76
65.90
66.24
70.77
76.12
76.70
77.02
77.22
77.34
123.94
125.11
128.90
134.00
139.62
145.46
207.90
207.87
ppm
20
40
60
80
100
120
140
160
180
200
ppm

b)

Appendices

```
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PROCNO              1

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Time              14 01
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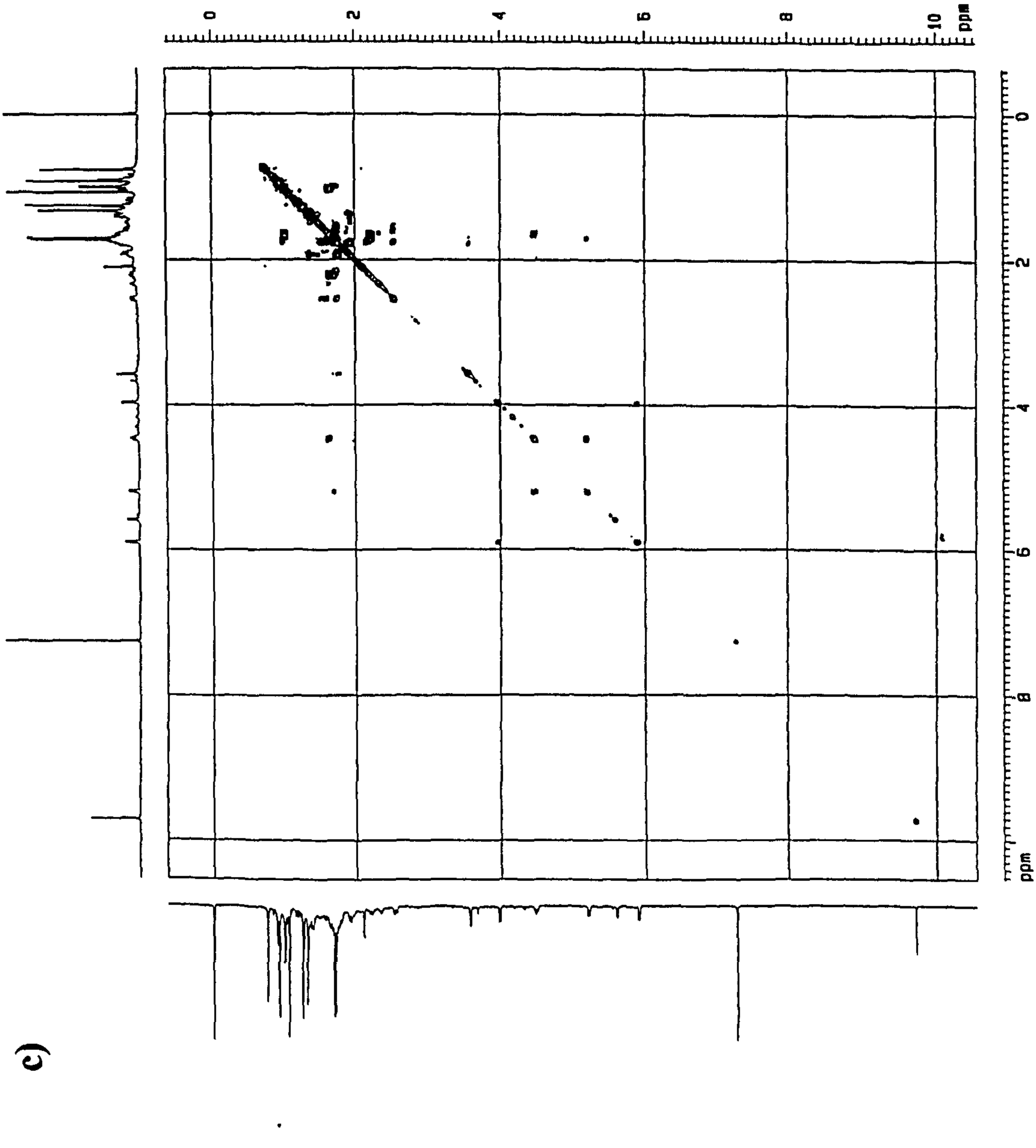
SOLVENT           CDCl3
AQ          0.2293960 sec
DW            112 0 usec
RG                1024
NUCLEUS           1H
H4.1                2 08
D1          1 0184330 sec
P1              9 5 usec
D8          0 0000030 sec
DE              172 9 usec
SFO1        400 1363731 MHz
SWH         4464 29 Hz
TD                2048
NS                   8
DS                   4
INO         0.0002240 sec

                F1 - Acquisition parameters
NDO                  1
TD                 256
SFO1        400.1364 MHz
FIDRES      17.438618 Hz
SW          11.157 ppm

                F2 - Processing parameters
SI                1024
SF          400.1343919 MHz
WDW               SINE
SSB                  0
LB           0 00 Hz
GB                   0
SR          4391 91 Hz
HZpPT       4 359654 Hz

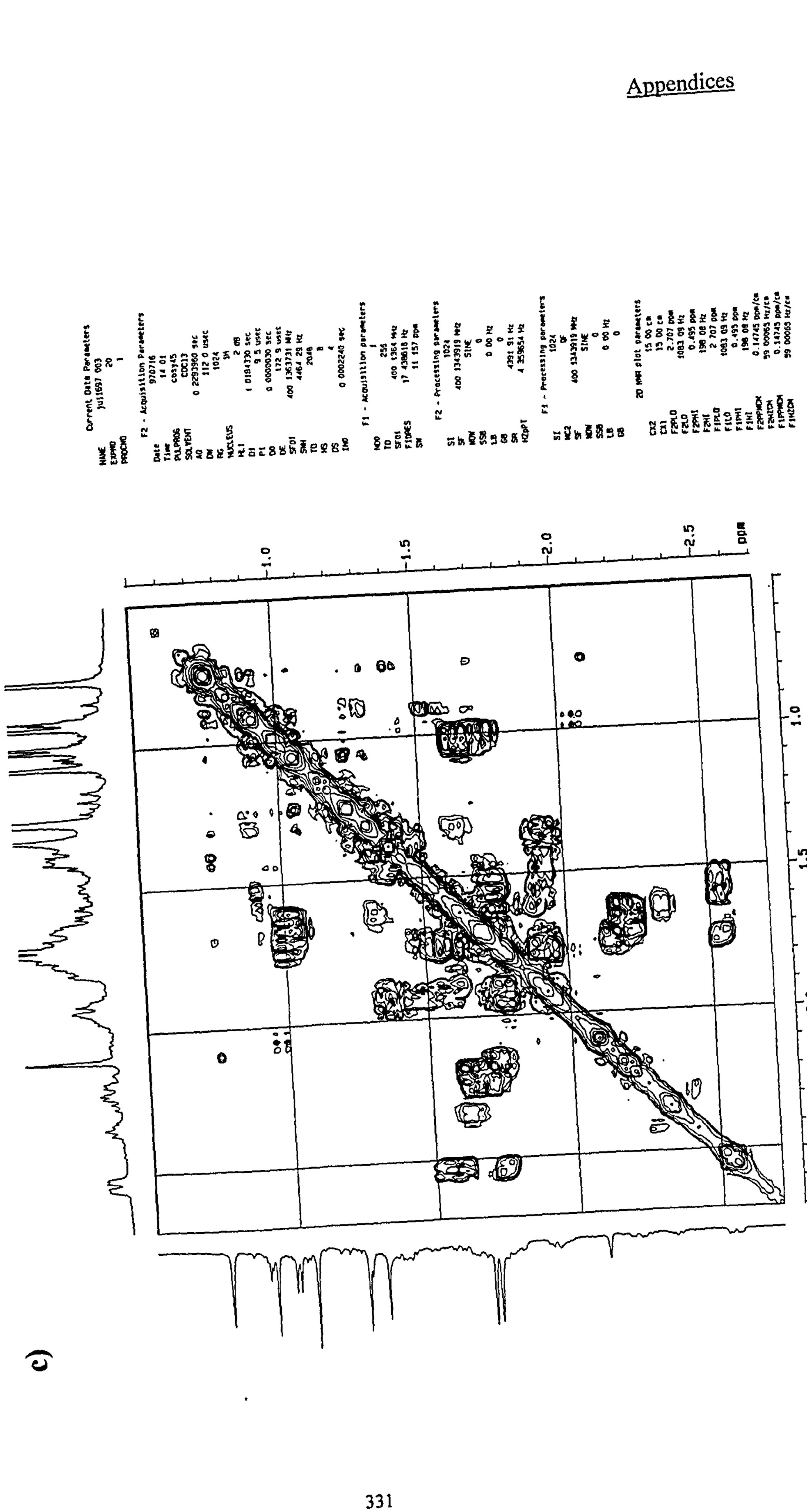
                F1 - Processing parameters
SI                1024
MC2                  IE
SF          400.1343919 MHz
WDW               SINE
SSB                  0
LB           0 00 Hz
GB                   0

                20 MHR plot parameters
CX2                 15 00 cm
CX1                 15 00 cm
F2PLO            10.530 ppm
F2LO            4213 31 Hz
F2PHI            -0.627 ppm
F2HI             -250 95 Hz
F1PLO            10.530 ppm
F1LO            4213 31 Hz
F1PHI            -0.627 ppm
F1HI             -250 95 Hz
F2PPMCM       0.74379 ppm/cm
F2HZCM        297 61758 Hz/cm
F1PPMCM       0.74379 ppm/cm
F1HZCM        297 61758 Hz/cm
```



c)

Appendices



c)

Appendices

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               34
                1

F2 - Acquisition Parameters
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             5 29
           noesytp
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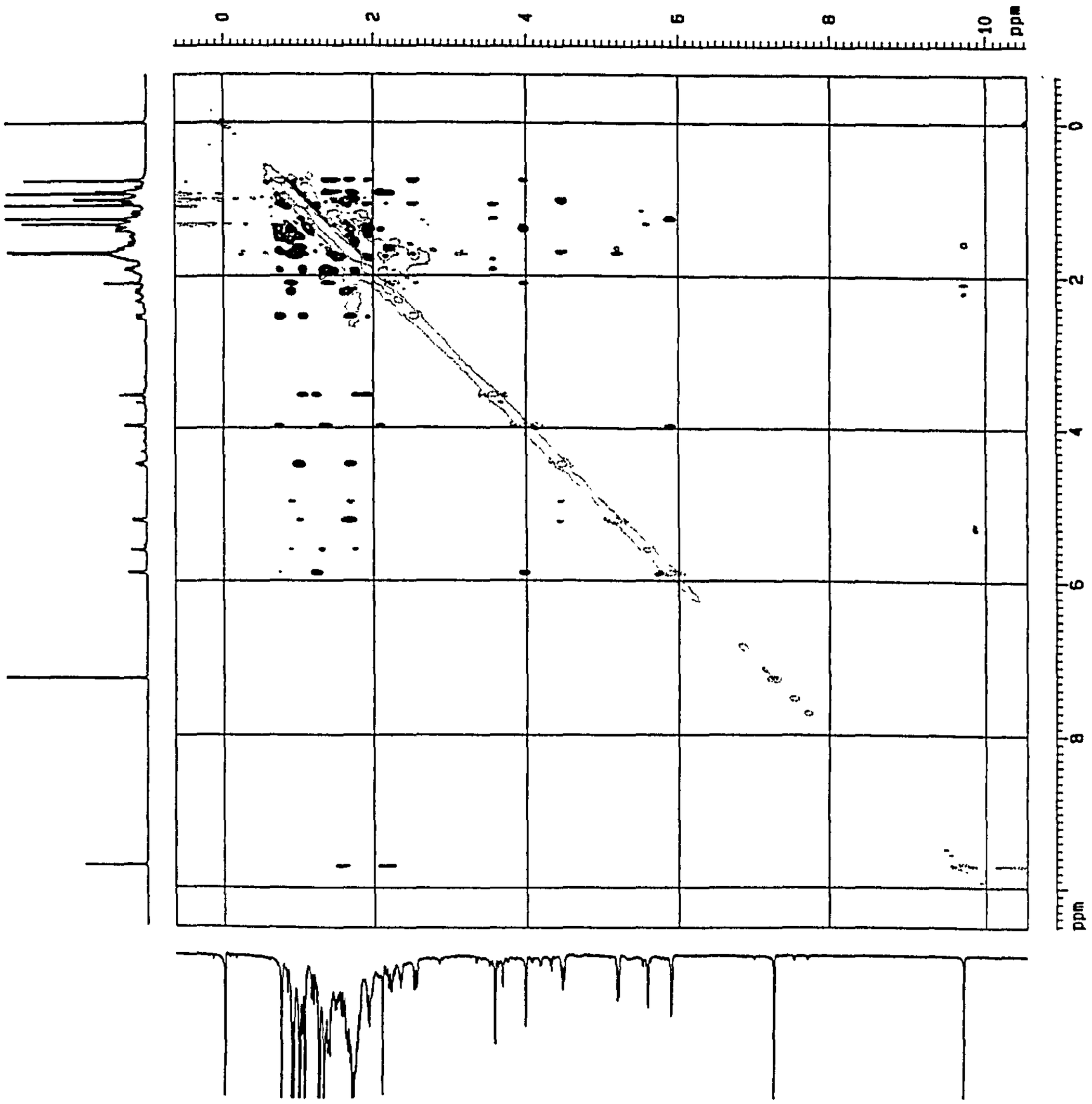
           112.0 usec
             1024
               1H
             2 dB
 0 7112638 sec
            9 5 usec
 0 0000030 sec
 0 6000000 sec
          175 7 usec
400 1363731 MHz
          4464.29 Hz
             1024
               32
                4
 0 0001120 sec

F1 - Acquisition parameters
                2
              256
          400.1364 MHz
          17 438616 Hz
          11 157 ppm

F2 - Processing parameters
              512
          400 1343919 MHz
               GSINE
                2
             0.00 Hz
                0
          4391 91 Hz
          8.719308 Hz

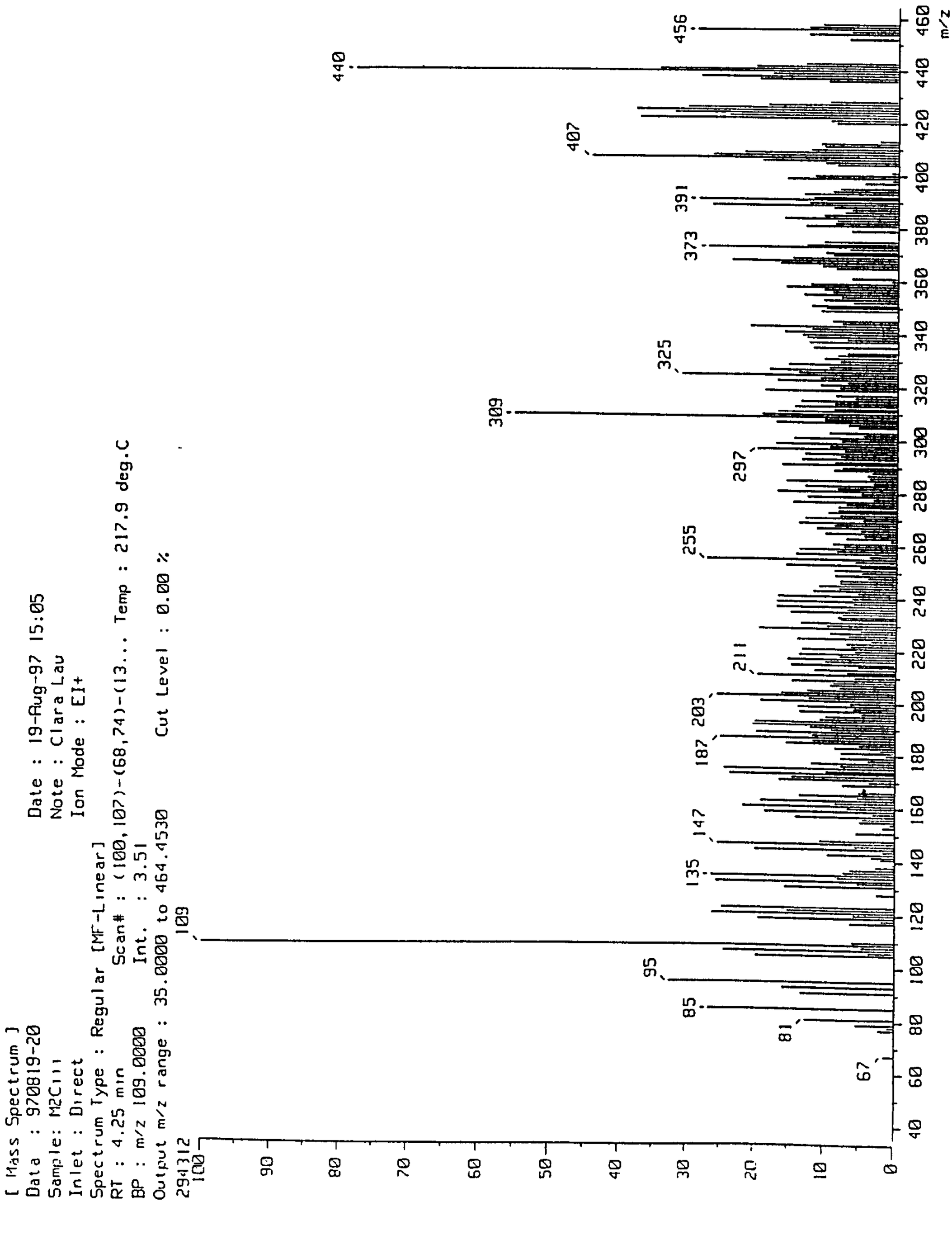
F1 - Processing parameters
              512
             TPPI
          400 1343919 MHz
               GSINE
                2
             0.00 Hz
                0

2D NMR plot parameters
          15.00 cm
          15.00 cm
          10.530 ppm
          4213 32 Hz
          -0 627 ppm
          -250.97 Hz
          10.530 ppm
          4213 32 Hz
          -0 627 ppm
          -250.97 Hz
          0 74380 ppm/cm
          297 61908 Hz/cm
          0 74380 ppm/cm
          297 61908 Hz/cm
```

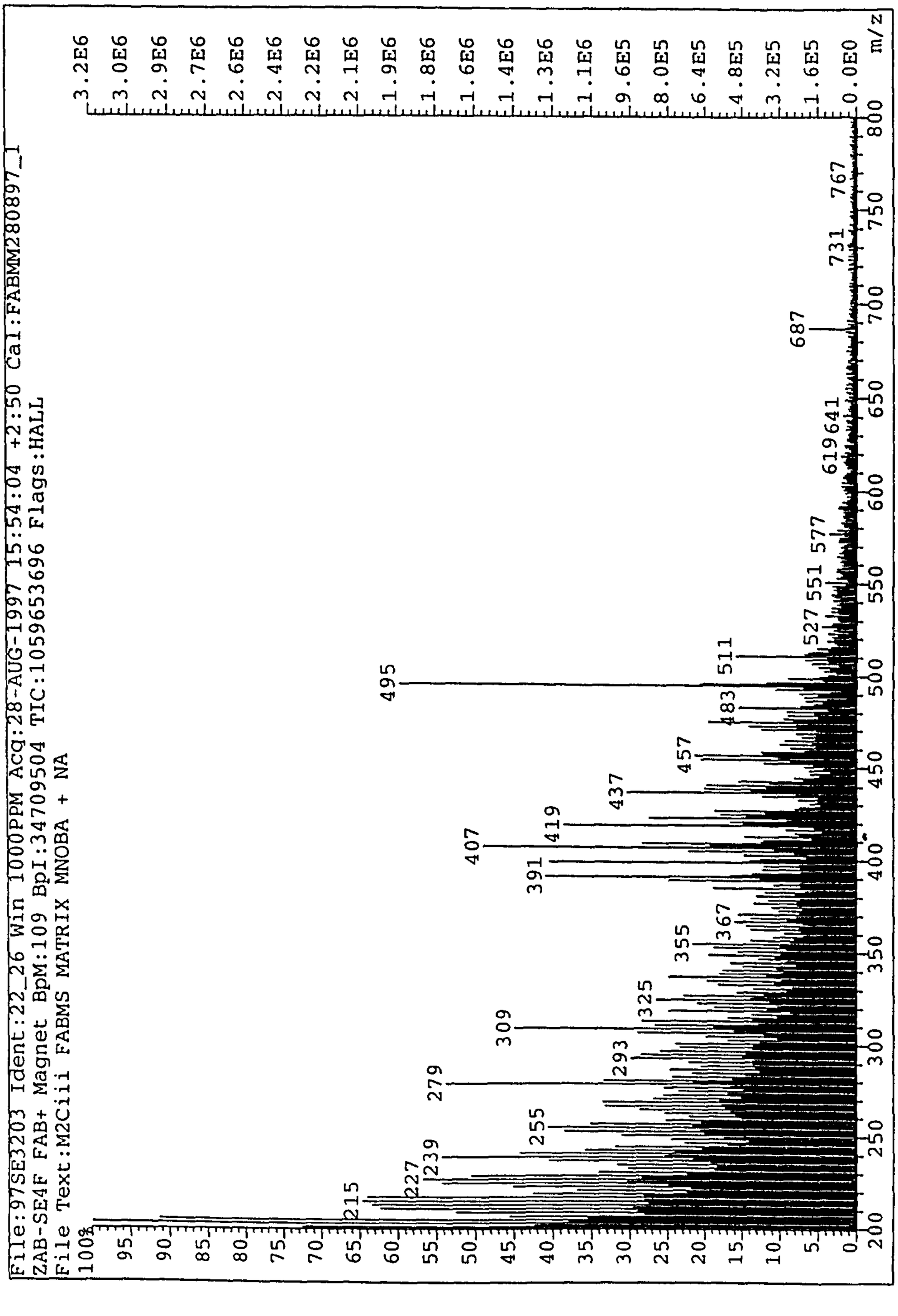


(d)

e)


[Mass Spectrum]
Data : 970819-20
Sample: M2C111
Inlet : Direct
Spectrum Type : Regular [MF-Linear]
RT : 4.25 min
BP : m/z 109.0000 Scan# : (100,107)-(68,74)-(13... Temp : 217.9 deg.C
Output m/z range : 35.0000 to 464.4530 Int. : 3.51 Cut Level : 0.00 %
2943112
109
95
85
81
67
135
147
187
203
211
255
297
309
325
373
391
440
456
m/z

f)


File:97SE3203 Ident:22_26 Win 1000PPM ACQ:28-AUG-1997 15:54:04 +2:50 Cal:FABMM280897_1
ZAB-SE4F FAB+ Magnet BpM:109 BpI:34709504 TIC:10596953696 Flags:HALL
File Text:M2Ciii FABMS MATRIX MNOBA + NA
100%
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
215
227
239
255
279
293
309
325
355
367
391
407
419
437
457
483
495
511
527
551
577
619
641
687
731
767
200
250
300
350
400
450
500
550
600
650
700
750
800
m/z
3.2E6
3.0E6
2.9E6
2.7E6
2.6E6
2.4E6
2.2E6
2.1E6
1.9E6
1.8E6
1.6E6
1.4E6
1.3E6
1.1E6
9.6E5
8.0E5
6.4E5
4.8E5
3.2E5
1.6E5
0.0E0

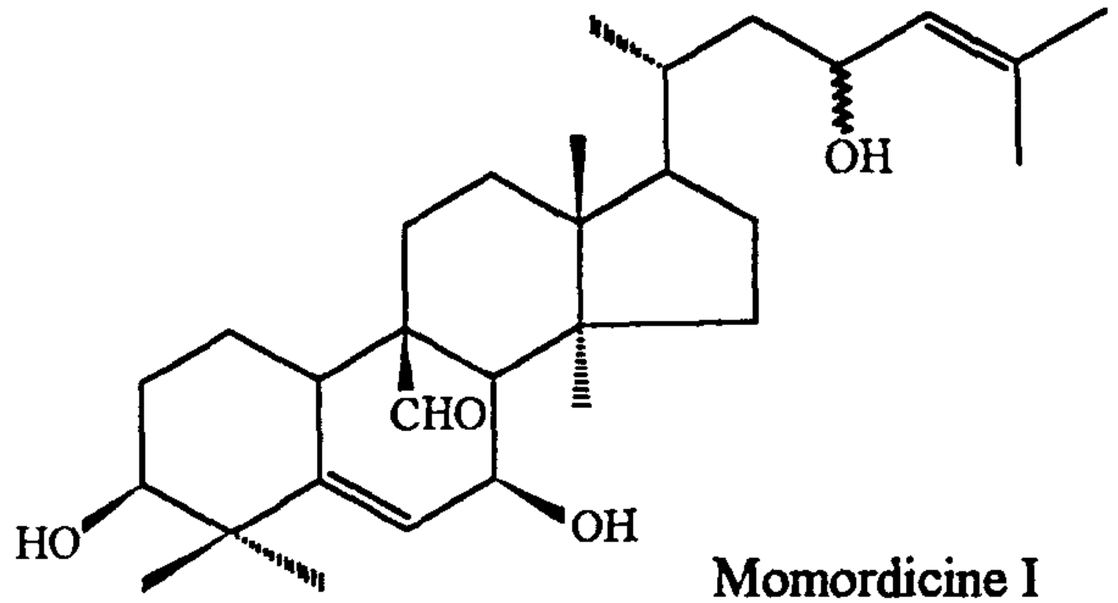
Appendix 13: NMR spectra for reference compound Momordicine I

a) ^{1}H 400 MHz NMR spectrum ($CDCl_3$)

b) ^{13}C 100 MHz NMR spectrum ($CDCl_3$)

N.B. Compound momordicine I obtained from Dr Hikaru Okabe, Fukuoka University, Japan.

a)



Momordicine I

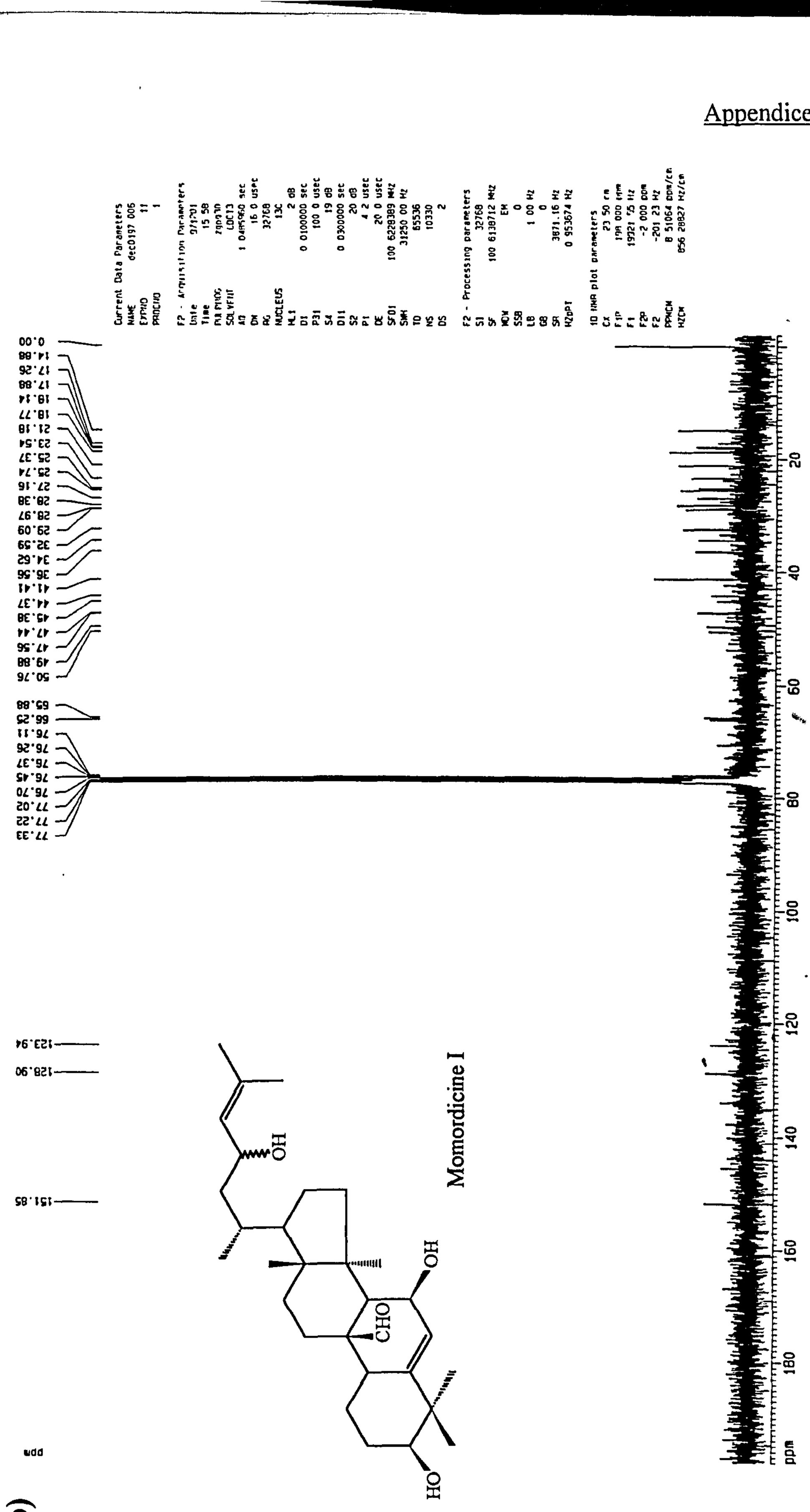
MY 1

Current Data Parameters
NAME dec0197 001
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date 971201
Time 11 02
PULPROG zg30
SOLVENT CDCl3
AQ 1 9661000 sec
DW 60 0 usec
RG 2048
NUCLEUS 1H
HL1 2 dB
D1 1 0000000 sec
P1 9 5 usec
DE 75 0 usec
SF01 400 1368820 MHz
SWH 8333 33 Hz
TD 32768
NS 16
DS 2

F2 - Processing parameters
SI 16384
SF 400 1343925 MHz
WDW EM
SSB 0
LB 0 30 Hz
GB 0
SR 4392 51 Hz
HZPT 0 508626 Hz

1D NMR plot parameters
CX 23 50 cm
F1P 10 600 ppm
F1 4241 42 Hz
F2P -0 400 ppm
F2 -160 05 Hz
PPMCM 0 46809 ppm/cm
HZCM 187 29694 Hz/cm


Current Data Parameters
NAME dec0197 006
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date 971201
Time 15 58
INSTRUM zgpg30
PROBHD LDC13
SOLVENT
AQ 1 0485960 sec
DW 16 0 usec
DE 32768
NUCLEUS 13C
NL1 2 dB
D1 0 0100000 sec
P31 100 0 usec
S4 19 dB
D11 0 0300000 sec
S2 20 dB
P1 4 2 usec
DE 20 0 usec
SFO1 100 6228389 MHz
SW4 31250 00 Hz
TD 65536
NS 10330
DS 2
F2 - Processing parameters
SI 32768
SF 100 6138712 MHz
WDW EM
SSB 0
LB 1 00 Hz
GB 0
SR 3871.16 Hz
HZPPT 0 953674 Hz
1D NMR plot parameters
CX 23 50 cm
F1P 199 000 ppm
F1 19921 75 Hz
F2P -2 000 ppm
F2 -201 23 Hz
PPMCM 8 51064 ppm/cm
HZCM 856 28827 Hz/cm
Title: MY 1
ppm
Momordicine I
OH
CHO
OH
OH
b)

Appendix 14: The product labels for the two commercially available karela capsules

a) Bought from "The Herb Room", Santa Cruz, USA. ($ 9.50 for 45 capsules)

b) Bought from "The Food Hall", Turnpike Lane, London N8, UK. (£4.99 for 30 capsules)